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(71) Applicant: MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US).

(72) Inventors: LEBOULCH, Philippe; Apartment 417, 10 Rogers Street, Cambridge, MA 02142 (US). LONDON, Irving, M.; 41 Fresh Pond Lane, Cambridge, MA 02138 (US). TUAN, Dorothy; 55 Cross Hill Road, Newton, MA 02159 (US).

(74) Agents: PABST, Patrea, L. et al.; Kilpatrick & Cody, Suite 2800, 1100 Peachtree Street, Atlanta, GA 30309-4530 (US).

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(57) Abstract

A process and means for the design and the optimization of retroviral vectors transducing human β-globin gene and β-Locus Control Region (β-LCR) derivatives, hereafter referred to as [β-globin/LCR] retroviruses, which successfully meet the following criteria required for gene therapy applications: (1) stability of proviral transmission (low frequency of rearrangements similar to retroviral vectors considered stable in the art) upon infection of cell-lines and murine bone marrow cells, (2) improved viral titer, thereby allowing successful infecti n of bone marrow cells, and (3) high erythroid expression of the transduced human β-globin gene, are described, along with specific constructs.

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RETROVIRAL VECTORS FOR TRANSDUCING BETA-GLOBIN GENE AND BETA-LOCUS CONTROL REGION DERIVATIVES

The present invention is in the field of molecular genetics and, in particular, relates to the field of retroviral vectors and methods for making these vectors for transducing ß-globin gene and ß-locus control region derivatives.

Background of the Invention

ß-thalassemias and sickle-cell anemia are human genetic disorders of the ß-globin gene, with severe clinical manifestations in homozygotes. At the present time, allogeneic bone marrow transplantation represents the best available possibility of cure for these patients. Unfortunately, few of them are able to locate a normal HLA-matched donor and even if a matched HLA donor were available, many would face severe complications of the bone marrow transplantation procedure such as graft versus host disease (Parkman, R., *Science* 232:1373-1378 (1986)). For these reasons, gene therapy using genetically modified autologous totipotent hematopoietic stem cells (THSC) is an attractive alternative to allogeneic bone marrow transplantation. Because gene targeting by homologous recombination is not yet technically possible in THSC, the most realistic strategy is to obtain stable integration of a normal human ß-globin gene and its cis-acting regulatory elements into the THSC genome. This can be achieved by retrovirus-mediated gene transfer, an efficient gene transfer technique applicable to these cells (Fraser *et al.*, *Blood*, 76:1071-1076 (1990)).

Gene transfer exp riments have previously shown that the proximal cis-acting elements of the human ß-globin gene are insufficient for gene

therapy applications because they provide a very low, integration site-dependent expression of the human ß-globin transgene (less than 1 to 5% of human ß-globin/murine$ß_{maj}$-globin mRNA ratio) (Cone *et al.*, *Mol. Cell Biol.* 7:887-897 (1987); Dzierzak *et al.*, *Nature* 331:35-41 (1988); Karlsson *et al.*, *Proc. Natl. Acad. Sci.* USA 85:6062-6066 (1988); Miller *et al.*, *J. Virol.*, 62:4337-4345 (1988); Bender *et al.*, *Mol. Cell. Biol.*, 9:1426-1434 (1989)). The discovery of major hypersensitive sites (HS) far upstream of the human β-globin gene locus, constituting the β-Locus Control region (β-LCR), has given new hope for successful gene therapy of human β-globin gene disorders. (Tuan and London, *Proc. Natl. Acad. Sci. USA* 81:2718-2722 (1984); Tuan *et al.*, *Proc. Natl. Acad. Sci. USA* 82:6384-6388 (1985); Forrester *et al.*, *Proc. Natl. Acad. Sci. USA* 86:5439-5443 (1989); Grosveld *et al.*, *Cell* 51:975-985 (1987)). LCR derivatives are able to confer erythroid-specific, high, integration site-independent expression of a linked ß-globin gene in transgenic mice and murine erythroleukemia (MEL) cells, which mimic adult erythroid differentiation (Grosveld *et al.*, *Cell* 51:975-985 (1987)). Because the activity of each HS site has now been localized to small DNA fragments (U.S. Patent No. 5,126,260; Curtin *et al.*, *Proc. Natl. Acad. Sci. USA* 86:7082-7086 (1989); Forrester *et al.*, *Proc. Natl. Acad. Sci. USA* 86:5439-5443 (1989); Ryan *et al.*, *Genes Dev.* 3:314-323 (1989); Tuan *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:2554-2558 (1989); Collis *et al.*, *EMBO J.*, 9:233-240 (1990); Ney *et al.*, *Genes Dev.* 4:993-1006 (1990); Philipsen *et al.*, *EMBO J.*, 9:2159-2167 (1990); Talbot *et al.*, *EMBO J.*, 9:2169-2178 (1990); Pruzina *et al.*, *Nucleic Acids Res.*, 19:1413-1419 (1991); Walters *et al.*, *Nucleic*

Acids Res., 19:5385-5393 (1991)), it has become possible to construct retroviral vectors transducing ß-LCR derivatives linked to the human ß-globin gene and its proximal cis-acting elements (Novak *et al.*, *Proc. Natl. Acad. Sci., USA*, 87:3386-3390 (1990); Chang *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:3107-3110 (1992)). However, these [ß-globin/LCR] retroviruses have low titer, are very unstable with multiple rearrangements upon transmission of the proviral structure, and provide a relatively modest and highly variable enhancement of ß-globin gene expression in infected murine erythroleukemia (MEL) cells (Novak *et al.*, *Proc. Natl. Acad. Sci. USA* 87:3386-3390 (1990); Chang *et al.*, *Proc. Natl. Acad. Sci. USA* 89:3107-3110 (1992)).

U.S. Patent No. 5,126,260 describes DNAaseI hypersensitive sites that constitute the β-LCR and, in particular, identifies the HS2 enhancer within the β-LCR structure. U.S. Patent No. 5,126,260 also claims the use of β-LCR and HS2 derivatives in gene transfer protocols, including retrovirus-mediated gene transfer, to obtain high expression level of the human β-globin gene. However, U.S. Patent No. 5,126,260 does not identify specific means by which stable proviral transmission of [β-globin/LCR] retroviruses can be achieved.

It is therefore an object of the present invention to provide retroviral vectors for stable transduction of the ß-globin gene and ß-locus control region derivatives and other erythroid specific genes.

Summary of the Inventi n

Retroviral vectors capable of transducing the human ß-globin gene and ß-Locus Control Region (ß-

LCR) derivatives, hereafter referred to as the [ß-globin/LCR] retroviral vectors, are provided. The [ß-globin/LCR] retroviral vectors successfully meet the following criteria required for successful gene therapy: (1) stability of proviral transmission, or low frequency of rearrangements similar to retroviral vectors considered stable in the art, upon infection of cell-lines and murine bone marrow cells; (2) improved viral titer, thereby allowing successful infection of bone marrow cells; and (3) high erythroid expression of the transduced human ß-globin gene, defined herein as greater than 50% of human ß-globin to murine β_{maj}-globin mRNA ratio on a per gene basis in pools of infected and dimethylsulfoxide-induced (DMSO-induced) murine erythroleukemia (MEL) cells.

Specific constructs that meet the criteria presented above are described in detail below.

Specific means to design additional [ß-globin/LCR] retroviral vectors meeting these criteria are also described.

The improved vectors are useful in the treatment of a variety of disorders including ß-thalassemia and sickle-cell anemia.

Brief Description of the Drawings

Figure 1 is a schematic representation of the general design of the [ß-globin/LCR] retroviral vectors of the present invention.

Figures 2A-D are reproductions of a computer analysis of Southern blots showing proviral transmission of the [ß-globin/LCR] retroviruses using a Neo^R specific probe. Figure 2A shows multiple rearrangements in cell lines infected with a [ß-globin/(HS2+HS3+HS4)/PGK] virus. Genomic DNA from infected cells was digested with *Sac*1. Lane P

depicts genomic DNA from cells infected with pool of producers. The right lanes depict genomic DNA from cells infected with independent producer clones. The expected position of correct provirus is indicated by an arrow. Figure 2B shows stable proviral transmission in infected cell lines obtained with [ß-globin/LCR]^{mut} vectors. Genomic DNA and control plasmids were digested with *Sac*1. The left lanes depict plasmids as size controls wherein lane 1 is [ß-globin/HS2/PGK]^{mut}; lane 2 is [ß-globin/(HS2+HS3+[4xCP2 HS4])/PGK]^{mut}; lane 3 is [ß-globin/(HS2+HS3+HS4)/PGK]^{mut}; lane 4 is [ß-globin/HS2/PGK/Neo^{R}-]^{mut}; lane 5 is [ß-globin/(HS2+HS3+[4xCP2 HS4])/PGK/Neo^{R}-]^{mut}; lane 6 is [ß-globin/(HS2+HS3+HS4)/PGK/Neo^{R}-]^{mut}. The right lanes show genomic DNA from infected cells. A pool of producers were used to infect cells in lanes 1, 2, 3, and 4, whereas single producer clones were used to infect lanes 5, 6 and 7. Lane 1 is [ß-globin/HS2/SV40]^{mut}; lanes 2, 5, 6, and 7 are [ß-globin/HS2/PGK]^{mut}; lane 3 is [ß-globin/(HS2+HS3+[4xCP2 HS4])/PGK]^{mut}; and lane 4 is [ß-globin/(HS2+HS3+HS4)/PGK]^{mut}. A minor rearranged component is present in lane 3. Figure 2C shows a Southern blot aimed at detecting micro-rearrangements within the transduced DNA insert by digesting genomic DNAs with *Sma*1, which is located in both LTRs as well as between LCR derivatives and the internal PGK/NeoR cassette. Lanes 1 to 7 are identical to the right lanes 1 to 7 of Figure 2B, except that DNAs were digested with *Sma*1 instead of *Sac*1. The arrow labelled "a" shows the correct position for lane 1 because there is no *Sma*1 site upstream of the SV40 promoter. The arrow labelled "b" shows an abnormal band in lane 3 corresponding to the minor rearranged form described above. The arrow labelled "c" shows the correct proviral

structure for constructs transducing the PGK promoter. Figure 2D shows proviral transmission following bone marrow transplantation in mice. Genomic DNAs from infected spleens (13 days post-engraftment) and from cell-line controls were digested with *Sac*1. Left lanes 1 and 2 contain size controls corresponding to lanes 1 and 3 of Figure 2B. Right lanes HS2 (1, 2, 3) show the results of three mice transplanted with [ß-globin/HS2/PGK]^{mut} obtained from a pool of viral producers. Right lanes Zen (1, 2, 3) show the results of three mice transplanted with Zen control virus. Lanes HS2+HS3+[4xCP2 of HS4] (1, 2) show the results of two mice transplanted with [ß-globin/(HS2+HS3+[4xCP2 HS4])/PGK]^{mut} from a pool of viral producers.

Figures 3a-3b are a DNA sequence analysis of [ß-globin/LCR] constructs, screening for the presence of potential deleterious sequences such as 5′ splice-sites (5′SS), 3′ splice-sites (3′SS) and branchpoint sites (BPS), and polyadenylation signals (polyA). Matches or mismatches with consensus sequences are indicated by capital letters or lowercases, respectively. Regions mutated by site-directed mutagenesis or related procedures are indicated by an asterisk.

Figures 4A-F are schematic representations of the steps involved in the mutagenesis procedure used to produce the retroviral constructs of the present invention. The numbering system corresponds to that of Figures 3a-3b and 5a-5c. Figure 4A shows PCR mediated deletion of the 372 bp [*Rsa*1-*Rsa*1] fragment in Intron 2. Two independent PCR reactions were performed using two pairs of primers overlapping the intragenic *Eco*R1 (1908), *Rsa*1 (2345), *Rsa*1 (2717) and *Bam*H1 (2820) sites, respectively. In order to tag the coding region of

the gene for further studies, mutations substituting two amino acids of β- with δ-globin amino acids were introduced in the *Eco*R1 primer. PCR products were digested with *Eco*R1, *Bam*H1, and *Rsa*1. A triple ligation was subsequently performed with the parental [ß-globin] vector opened at *Eco*R1 and *Bam*H1 sites. Figure 4B shows that region [1665 to 1770] was reconstituted and mutated by ligation of four complementary and overlapping oligonucleotides with the LXSN vector opened at *Eco*R1 and *Xba*1 sites. A polylinker was included in the oligonucleotide sequence, to prepare for subsequent steps of the construction. Only two of the four oligonucleotides were phosphorylated to prevent concatemerization upon ligation. The ligation product was digested with *Xho*1 prior to transformation to eliminate parental plasmid. Figure 4C shows that region [1770 to 2185] was reconstituted and mutated by PCR mediated construction. Point mutations and additional restriction sites (*Mlu*1 and *Hind*3) were introduced in PCR primers. These new sites allowed ligation with the vector obtained in the step shown in Figure 4B opened at *Mlu*1 and *Hind*3 sites. The ligation product was digested with *Bam*H1 prior to transformation to eliminate parental plasmid. Figure 4D shows that region [2185 to 3250] was reconstituted and mutated by PCR mediated construction, with an approach similar to the step shown in Figure 4C. The template used contained the 372 bp intronic deletion obtained in the step shown in Figure 4A. After cutting the vector and PCR fragment with *Sac*1 and *Nco*1, ligation was performed with a vector containing HS2, the ß-globin promoter and the first part of the gene. The ligation product was digested with *Sma*1 prior to transformation to eliminate parental plasmid.

Figure 4E shows that the [*Hind*3-*Bgl*2] insert from the construct shown in Figure 4D was ligated with the backbone of the construct shown in Figure 4C opened with *Hind*3 and *Bgl*2. The ligation product was digested with *Cla*1 prior to transformation to eliminate parental plasmids and unwanted forms. Figure 4F shows that the final [ß-globin/HS2]^{mut} retroviral construct was obtained by ligating a [*Bgl*1-*Bgl*2] fragment from the construct shown in Figure 4E with a [*Bgl*2-*Bgl*1] fragment containing an enhancer/promoter/NeoR cassette and a [*Pvu*2-*Xba*1] deleted MoMLV LTR. The ligation product was digested with *Apa*1 prior to transformation to eliminate parental plasmids and unwanted forms. Accuracy of the construct was verified by DNA sequencing.

Figures 5a-5c are the DNA sequence (Sequence Listing ID No. 1) of the retrovirally transduced human ß-globin gene in C/R orientation, from base 1665 to 3325 according to the numbering system set forth in Figures 3a-3b. The three exons, two introns, and promoter are indicated. The 372 bp [*Rsa*1-*Rsa*1] deletion in Intron 2 is underlined. Mutated PolyA and 3'SS are indicated. Point mutations introduced by mutagenesis are shown below the wild-type sequence. Codons maintained or substituted by corresponding codons from human *δ*-globin gene are indicated. Restrictions sites relevant to the mutagenesis are boxed and numbered. Oligonucleotides used for the mutagenesis are represented by arrows.

Figures 6A-D are reproductions of a computer analysis of RNA protection assays. The left and right tracks of each lane contain a murine specific ß-globin probe and a human specific ß-globin probe, respectively. Positions of sp cific protected fragments are indicat d by lower case letters to the right of

each blot. The band for human ß- is "a", murine $ß_{maj}$- is b, and murine $ß_{min}$- is c. Left lanes M and H of Figure 6A show positions of undigested murine (M) and human (H) probes. Lane 1 is RNA extracted from a pool (> 100) of electroporated (average of three copies per cell), G418 selected, and DMSO induced MEL cells, using [ß-globin/HS2/PGK] construct. Lanes 2 to 5 are RNA extracted from a pool (> 100) of infected (one provirus per cell), G418 selected, and DMSO induced MEL cells, wherein lane 2 is [ß-globin/HS2/SV40]^{mut}, lane 3 is [ß-globin/HS2/PGK]^{mut}, lane 4 is [ß-globin/(HS2+HS3+[4xCP2 HS4])/PGK]^{mut}, and lane 5 is [ß-globin/(HS2+HS3+HS4)/PGK]^{mut}. Figure 6B is a reproduction of RNA protection assays using RNA extracted from cells obtained by *in vitro* clonogenic assays following infection of murine bone marrow cells with [ß-globin/HS2/PGK]^{mut}. Figure 6C shows the effect of heterologous enhancers wherein RNA was extracted from a pool (> 100) of infected (one provirus per cell), G418 selected, and DMSO induced MEL cells and wherein lane 1 is [ß-globin/(HS2+[4x23bp HS2])/F441 Py]^{mut}, lane 2 is [ß-globin/HS2/F441 Py]^{mut}, lane 3 is [ß-globin/HS2/SV40]^{mut}, lane 4 is [ß-globin/HS2/PGK]^{mut}, and lane 5 is [ß-globin/SV40]^{mut}. Figure 6D shows RNA protection assays aimed at measuring position-dependent variability of expression. Lanes 1 to 6 show RNA extracted from individual clones of infected (one provirus per cell), G418 selected, and DMSO induced MEL cells, using the [ß-globin/HS2/PGK]^{mut} construct.

Detailed Description of the Invention

Retroviral vectors capable of transducing the human ß-globin gene and ß-Locus Control Region (ß-

LCR) derivatives (the [ß-globin/LCR] retroviral vectors), for the treatment of disorders such as ß-thalassemia and sickle-cell anemia by gene therapy, and methods for producing these vectors are provided. These [ß-globin/LCR] retroviral vectors are superior to currently available retroviral vectors in that they (1) exhibit stable proviral transmission with a low frequency of rearrangements upon infection of cell-lines and murine bone marrow cells, (2) produce higher viral titers, thereby allowing successful infection of bone marrow cells, defined herein as greater than 10^5 G418 resistant NIH 3T3 colonies per ml of viral supernatant under standard conditions, and (3) cause high erythroid expression of the transduced human ß-globin gene, defined herein as greater than 50% of human ß-globin to murine$_{maj}$-globin mRNA ratio on a per gene basis in pools of infected and dimethylsulfoxide-induced (DMSO-induced) murine erythroleukemia (MEL) cells.

Structures believed to be responsible for the proviral instability and low titer of [ß-globin/LCR] retroviral vectors currently available have now been identified. These structures include an A/T rich segment in the second intron of the human ß-globin gene, and several complementary/reverse (C/R) polyadenylation signals and splice-sites. Extensive mutagenesis of the transduced ß-globin gene that results in elimination of these structures renders proviral transmission stable upon infection of cell-lines and murine bone marrow stem cells, increases viral titer ten-fold, and does not significantly perturb the expression of the transduced ß-globin gene. The optimized retroviral vectors described herein have enabl d study of the expression properties of various retrovirally-transduced ß-LCR derivatives

in DMSO-induced MEL cells and achievement of greater than 50% of human ß-globin/murine$_{maj}$-globin mRNA ratios on a per gene basis. The question of position-independent expression following chromosomal integration has also been addressed. The influence of heterologous enhancers/promoters on the expression of the retrovirally transduced ß-globin gene, when cis-linked to ß-LCR derivatives, has also been analyzed.

As used herein, a retrovirus is an animal virus belonging to the virus family of Retroviridae, including any types, subfamilies, genus, or tropisms.

Retroviral vectors, in general, are described by Verma, I.M., Retroviral vectors for gene transfer. In MICROBIOLOGY-1985, American Society for Microbiology, pp. 229-232, Washington, 1985, which is incorporated by reference herein.

Identification of DNA sequences deleterious for stability and titer of [ß-globin/LCR] retroviruses.

The structures believed to be responsible for the proviral instability and low titer were identified by the following procedure. A computer search for DNA sequences within ß-globin gene and ß-LCR derivatives that could be potential causes of retroviral rearrangements was conducted. Two general mechanisms of retroviral instability have been previously described: (1) inappropriate splicing or polyadenylation creating deletions in the viral genomic RNA, and/or (2) rearrangements during the steps of reverse transcription often triggered by various types of repeated sequences. The computer search was therefore conducted for a virtual concatemer representing a 5 kb DNA segment composed, from 5′ to 3′, of the 814 bp hybrid xtended packaging signal (Ψ+); the 2748 bp fragment containing the human ß-globin gene and its

promoter, in complementary/reverse (C/R) orientation; the 374 bp HS2 fragment, in C/R orientation; the 287 bp HS3 fragment, in direct orientation; the 243 bp HS4 fragment, in C/R orientation; the 583 bp murine phosphoglycerate kinase-1 (PGK) promoter, in direct orientation. Elements upstream or downstream of this 5 Kb segment were not included in this search, because rearrangements in LTRs or markers for antibiotic resistance (Neo^R) would most likely not be compatible with viral transmission of G418 resistance.

Five C/R [AATAAA] cleavage/polyadenylation (polyA) signals were identified, all located in the ß-globin gene, as shown in Figures 3a-3b and 5a-5c. No strong downstream polyA element such as [T]n, [GT]n, or [YGTGTTYY] was detected for most of them, except for polyA at position 1704 in Figure 3a, which is followed by nine [T] in an 11 bp segment immediately downstream. For splicing signals, the consensus described in vertebrate animals reported by Krainer and Maniatis, (1988) In Hames, B., D., and Glover, D., M. (ed.), TRANSCRIPTION AND SPLICING. (IRL Press, Oxford) pp. 131-206):
$[C_{38}/A_{39}A_{62}G_{77}G_{100}T_{100}A_{60}A_{74}G_{84}T_{50}]$ for 5'SS,
$[Y_{77}Y_{78}Y_{81}Y_{83}Y_{89}Y_{85}Y_{82}Y_{81}Y_{86}Y_{91}Y_{87}NY_{97}A_{100}G_{100}]$ for 3'SS, and
$[Y_{13/16}NY_{16/16}T_{14/16}R_{13/16}A_{16/16}Y_{15/16}]$ for branchpoint sites (BPS), 2 to 21 bp upstream of a putative 3'SS. In addition, potential 5'SS were grouped according to the following five classes previously described by Krainer and Maniatis, *supra* (1988): class I = AGGTA; class II = GTAAG; class III = RGGTGAG; and class IV = AGGTNNGT.

Eighteen potential 5'SS with two mismatches or fewer, and thirty-two potential 3'SS with three mismatches or fewer were identified, as shown in Figures 3a-3b and 5a-5c. With regard to direct repeats, a

region very rich in A/T, including degenerated tandem direct repeats of motifs such as [AAAAT]n or the variant [AAAAN]n, was noted in Intron 2, as shown in Figures 5a-5c. Three of the C/R polyA sites are present in this A/T rich area, which has also been reported by Miller *et al.*, *J. Virol.* 62:4337-4345 (1988) to have a possible deleterious effect on the propagation of [ß-globin] retroviral vectors. Another extended direct tandem repeat [ATTTATATGCAGAAATATT] (Sequence Listing ID No. 2) was found in Intron 2, as shown in Figures 5a-5c. No homology was detected with constitutive retroviral elements such as Ψ+, tRNA primer binding site (PBS), or LTR including the integration (IN) motif. No strong homology with the polypurine track for RNAase H cleavage of viral genomic RNA and initiation of positive-strand strong-stop was detected, although two polypurine segments in Intron 2 were identified: [GGAGAAGAAAAAAAAAGAAAG] (Sequence Listing ID No. 3) and [AGAAAAGAAGGGGAAAGAAAA] (Sequence Listing ID No. 4), as shown in Figures 5a-5c. No extended inverted repeats were detected.

Description of specific [ß-globin/LCR] retroviral constructs useful for gene therapy.

The general design of the [ß-globin/LCR] retroviral vectors of the present invention is described in Figure 1. The ß-globin gene is inserted in reverse orientation with respect to the direction of transcription of the provirus, to prevent splicing of the ß-globin introns on the viral genomic RNA prior to reverse transcription. All the constructs in these examples are derived from the LXSN vector (Miller and Rosman, *BioTechniques*, 7:980-990 (1989), the teachings of which are incorporated by reference herein), however, any other retrovirus-based vector can be used.

The principal features of LXSN (Dusty Miller, The Fred Hutchinson Center, Seattle, WA) include from 5′ to 3′: the left LTR of Moloney murine sarcoma virus (MoMSV), the tRNA primer binding site (PBS) of MoMSV, a hybrid extended packaging signal (Ψ+) described below, a polylinker for DNA insertion, an internal SV40 enhancer/early promoter driving a NeoR gene, the polypurine track of Moloney murine leukemia virus (MoMLV), and the right LTR of MoMLV. Ψ+ extends into the gag region for production of high titer viruses. To prevent expression of MoMLV gp85 and p65 gag proteins, the p65 gag start codon of this vector is mutated into a stop codon and the upstream part of the vector (left LTR up to the 5′ part of Ψ+) is substituted with homologous sequences from MoMSV which does not express gp85 gag. This region also contains a hybrid intron with 5′SS from MoMSV and cryptic 3′SS from MoMLV.

The 5′ border of the human ß-globin promoter is the *Sna*B1 site 266 bp upstream of the CAP site. The 3′ border was optimized by removing most of the 3′ flanking region of the human ß-globin gene. Only 30 bp downstream of the gene were retained, to allow normal cleavage/polyadenylation of the human ß-globin gene. The right LTR was kept intact, or a self-inactivating vector was designed, by creating in the 3′ LTR of LXSN, a 176 bp [*Pvu*2-*Xba*1] deletion described previously in pZipNeoSV(X)1 by Cone *et al.*, *Mol. Cell Biol.* 7:887-897 (1987) and Dzierzak *et al.*, *Nature* 331:35-41 (1988), the teachings of which are incorporated by reference herein.

With respect to the LCR derivatives, all constructs contain a 374 bp [*Hind*3-*Xba*1] fragment containing the HS2 enhancer described in U.S.

Patent No. 5,126,260, the teachings of which are incorporated herein.

In addition to the HS2 enhancer, one construct contains a 287 bp HS3 fragment obtained by PCR starting 21 bp upstream [AGACCCT ...] and ending 41 bp downstream [... CCTATAC] of the 225 bp [*Hph*1-*Fnu*4H1] HS3 core described by Philipsen *et al.*, *EMBO J.* 9:2159-2167 (1990) and a 243 bp HS4 fragment obtained by PCR starting 27 bp downstream [GGGTATA ...] and ending at the *Ava*1 site of the 280 bp [*Sst*1-*Ava*1] HS4 core fragment described by Pruzina *et al.*, *Nucleic Acid. Res.* 19:1413-1419 (1991). Another construct contains only the above described HS4 fragment next to the HS2 enhancer fragment, without HS3.

SV40 enhancer/early promoter driving NeoR of LXSN was substituted by the F441 Py enhancer/TK promoter/NeoR cassette of PMC1neo (Stratagene, San Diego, CA) or a murine phosphoglycerate kinase (PGK-1) promoter/NeoR cassette (obtained from Rudolf Jaenisch, The Whitehead Institute, Cambridge, MA). In some constructs, the heterologous enhancer/NeoR cassette was deleted, to increase viral titer, although no selection was now possible.

Selection markers that can be inserted into the retroviral vectors include in addition to the neomycin/G418 resistance gene, a hygromycin resistance gene, a puromycin resistance gene, a phleomycin resistance gene, a dihydrofolate reductase gene, and a multidrug-resistance gene. Other markers that can be used include any molecule, such as the gene encoding ß-galactosidase, which interacts with a substrate to produce a colored cell, or a molecule expressed at the c ll membran and used in a cell sorting

procedure, for example by interaction with a specific antibody.

Characteristics of the modified transduced ß-globin gene and ß-LCR derivatives

The foregoing modifications of the transduced ß-globin gene and ß-LCR derivatives caused increased viral titer, restored stability of proviral transmission in cell-lines and bone marrow stem cells, and did not impair expression of the transduced ß-globin gene.

Initially, the removal of DNA segments potentially deleterious for titer and stability, which were likely to be neutral for ß-globin gene expression, was attempted. A 372 bp fragment of Intron 2 between two *Rsa*1 sites respectively located at + 580 and + 952 from the human ß-globin cap site was deleted. This deleted fragment contains most of the satellite DNA-like A/T rich segment, three of the five potential polyA sites, and one of the polypurine tracks. This deleted segment is clearly distant from essential intronic structures such as normal 5'SS, 3'SS, branchpoint, and intragenic enhancer. Since *Rsa*1 sites are frequent cutters, this deletion was performed by recombinant PCR, as shown in Figures 4A and 5a-5c.

The results indicate that the 372 bp deletion in Intron 2 and the shortening of the 3' flanking region increase viral titer of [ß-globin/LCR] constructs by ten-fold (Table I), but is not capable in itself to prevent instability of proviral transmission in the presence of complex ß-LCR derivatives such as [HS2 + HS3 + HS4].

Table I: Comparison of transmission and expression properties of [ß-globin/LCR] retroviruses.

Type	human ß[a] Murine $ß_{maj}$ + $_{min}$	human ß[a] Murine $ß_{maj}$	Viral Titer[b]
pZipneoSV(X)ß-globin (RO) enh+c	2%	5%	2×10^4
[ß-globin/HS2/SV40][d]	27%	63%	10^4
$[ß\text{-globin/SV40}]_{mut}$	11%	27%	2×10^5
$[ß\text{-globin/HS2/SV40}]_{mut}$	26%	69%	10^5
$[ß\text{-globin/HS2/F441 Py}]_{mut}$	32%	73%	10^5
$[ß\text{-globin/HS2/PGK}]_{mut}$	29%	70%	4×10^5
$[ß\text{-globin/(HS2+[4x23bp HS2])/PGK}]_{mut}$	18%	46%	2×10^5
$[ß\text{-globin/(HS2+HS3+[4xCP2 HS4])/PGK}]_{mut}$	36%	76%	2×10^4
$[ß\text{-globin/(HS2+HS3+HS4)/PGK}]_{mut}$	38%	82%	10^4

a Pool of greater than 100 G418 resistant MEL cell clones. Corrected values for specific activities of the probes and on a per gene basis (one proviral copy per cell in pseudo-diploid MEL cells)

b Titers of best Ψcre producers, measured by transmission of G418 resistance to NIH 3T3 cells (cfu/ml).

c Construct provided by R. Mulligan, Whitehead Institute and MIT, Cambridge, MA, and described by Cone, et al., *Mol. Cell Biol.* 7:887-897 (1987) and Dzierzak, et al., *Nature* 331:35-41 (1988)

d Deletion of part of the 3' flanking region of transduced human ß-globin gene was observed with this construct.

A more extended deletion of 774 bp in Intron 2 was also performed, with similar results. The sequence of this new intron, constructed by oligonucleotide-mediated construction, is CTGTGGGAGGAAGATAAGAGGGATGAACATGATTAGCAAAAGGGCCTAGCT TGGACGCGTCATCA AGGGTCCCATAGACTCAC (represented in complementary/reverse orientation; bases substituted or introduced are underlined) (Sequence Listing ID No. 5).

In an additional effort to stabilize proviral transmission of [ß-globin/LCR] retroviral vectors, extended site-directed mutagenesis was performed to eliminate other complementary/reverse potential SS and polyA signals. Because there were indications that most of the rearrangements occurred in the transduced ß-globin gene, the focus was directed toward potential SS sites located in the ß-globin gene itself, and the first phase of mutagenesis limited to 3'SS. In this process, care was taken not to alter known cis-acting features and coding regions. Accordingly, seven of the ten potential C/R 3'SS localized in the ß-globin gene were destroyed by point mutation in [AG], as shown in Figures 5a-5c. In particular, all the potential 3'SS presenting one or two mismatches were mutated. In addition, one point mutation was created in the polyA at position 1704, as shown in Figures 3a-3b, which is followed by a strong downstream [GT]/[T] region. A total of twenty point mutations were introduced in this first phase of the mutagenesis, by a complex multistep construction procedure described in Figure 4.

These additional mutations restored stability of proviral transmission in cell-lines and bone marrow stem cells, and did not impair expression of the transduced ß-globin gene. These optimized [ß-

globin/LCR] retroviruses are ref rred h reafter as to [ß-globin/LCR]^{mut}.

In order to stabilize further proviral transmission in some constructs, the 340 bp [*Bam*H1-*Xho*1] fragment of pZipNeoSV(X)1 containing the 3'SS of Moloney MLV used to generate the sub-genomic "ENV" transcript was inserted in some constructs. This fragment appeared either neutral, useful or deleterious depending on the construct used.

Stability of proviral transmission upon infection of cell-lines and murine bone marrow stem cells.

Proviral transmission of [ß-globin/LCR]^{mut} viruses was tested upon infection of NIH 3T3 and MEL cells with supernatant from producers generated with these constructs. Southern blot analysis of genomic DNA from infected and G418 selected cells demonstrated stable proviral transmission with all constructs, even in the presence of [HS2 + HS3 + HS4] derivatives and when infection was performed with pool of producers, as shown by Figure 2B. Only a minor rearranged component (less than 10%) was detected in a pool of infected cells generated from viruses containing (HS2 + HS3 + [4 x CP2 of HS4]). When independent producer clones were analyzed, most expressed a non-rearranged form. To detect microrearrangements that could have escaped this analysis, genomic DNA from infected and G418 selected cells was digested with *Sma*1, which is located in both LTR's as well as between ß-LCR derivatives and the internal PGK/NeoR cassette. The internal *Sma*1 site was always conserved, confirming the absence of microdeletion in this proviral region, as shown by Figure 2C. Although Novak *et al.*, *Proc. Natl. Acad. Sci., USA* 87:3386-3390 (1990) and Chang *et al.*, *Proc. Natl. Acad. Sci., USA* 89:3107-3110 (1992) have reported that

[ß-globin/LCR] retroviral constructs have the propensity to undergo additional rearrangements after multiple passages of producers, no such rearrangements were observed, even after several weeks of continuous culture.

To further challenge the stability of mutated vectors, an intact right LTR was introduced in the [ß-globin/HS2/PGK]^{mut} construct, and a "ping-pong" infection was performed by co-cultivation of amphotropic Ψcrip and ecotropic Ψcre producers for two weeks. No rearrangement was observed following this challenge. Packaging cells Ψcre and Ψcrip (Danos and Mulligan, *Proc. Natl. Acad. Sci. USA*, 85:6460-6464 (1988)), provided by Richard Mulligan (Whitehead Institute and MIT, Cambridge, MA) were grown at 37°C with 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 100 IU/ml penicillin and 100 mg/ml streptomycin. Plasmid DNAs used for transfection were prepared by the Quiagen procedure, according to protocol provided by the manufacturer (Quiagen, Inc., Chatsworth, CA). Because self-inactivating vectors were used, plasmid DNAs were directly transfected in packaging cells using a calcium phosphate procedure (5prime:3prime, Inc), after linearization of the plasmids outside the proviral structure (*Nde*1 site). Following G418 selection (500 mg/ml of active fraction) (Gibco, BRL, Gaithersburg, MD), pool of producers or independent producer clones were isolated and expanded. In the case of "ping-pong" experiments using non-self-inactivating vectors, Ψcre and Ψcrip producers were mixed and co-cultivated for 10 days. Producer cells were used directly for infection by co-cultivation, or viruses were prepared by filtration of supernatant through 0.45 mm Millipore filter, as

described by Danos and Mulligan, *Proc. Natl. Acad. Sci. USA* 85:6460-6464 (1988).

Viral titers obtained with these vectors are presented in Table I above. To test whether [ß-globin/LCR]^{mut} vectors are able to transmit correct proviral structure to hematopoietic stem cells, murine bone marrow cells were infected with pool of producers from 5-fluorouracil (5-FU) treated donor mice. Two constructs were chosen: [ß-globin/HS2/PGK]^{mut} because of higher titer, and [ß-globin/(HS2 + HS3 + [4 x CP2 of HS4])/PGK]^{mut} because the minor rearranged component observed with this construct could indicate a tendency for instability and would represent additional challenge. An "empty" Zen vector (titer greater than 10^6/ml) (described by Fraser *et al.*, *Blood*, 76:1071-1076 (1991)) was used as a control. Infected bone marrow was plated for *in vitro* clonogenic assays to estimate gene transfer efficiency, or was transplanted into lethally irradiated syngeneic mice. Gene transfer efficiency was estimated by comparing the number of macroscopic CFU-Mix-Erythroid colonies in the presence or in the absence of G418.

Bone marrow cells were isolated from adult male (C57BL/6J x C3H/HeJ)F1 mice injected intravenously four days previously with 5-FU (150 mg/Kg). For infection, 6 x 10^6 bone marrow cells were added to 90% confluent irradiated (15 Gy x-ray) viral producer cells in 100 mm Petri dishes in α-medium containing 10% FCS, 5% CS (for Ψcre derived ß-globin viral producers) or 5% newborn calf serum (for GP-E86-derived JZenNeo viral producers), 5% pokeweed mitogen-stimulated spleen cell conditioned medium, 100 ng/ml murine Steel factor (Immunex, Seattle, WA) and 4 µg/ml Polybrene™ (Sigma, St. Louis, MO). Co-cultivation

were performed for two days, with or without selection (prior to assay) in G418 (500 (active) μg/ml) for one additional day. Non-adherent and adherent cells, recovered by trypsinization, were combined for clonogenic progenitor assays or transplantation into irradiated (9.5 Gy ^{137}Cs) recipient mice. Transplant recipients received 2 x 10^6 pre-infection cell equivalents intravenously and were sacrificed three weeks later for DNA isolation from the spleen. For clonogenic progenitor assays, cells were plated at 1.5 x 10^4 pre-infection cell equivalents in 35 mm Petri dishes in 1.1 ml of a culture medium containing 0.8% methylcellulose, 30% FCS, 1% bovine serum albumin, 10^{-4}M ß-mercaptoethanol, 3 units/ml human urinary erythropoietin, 2% Pokeweed mitogen-stimulated spleen cell conditioned medium and 10% agar stimulated human leukocyte conditioned medium (Media Preparation Service, Terry Fox Laboratory, Vancouver, Canada), with or without G418 (0.8 (active) μg/ml). After 18 days incubation, macroscopic-erythroid colonies were scored by standard criteria described by Humphries *et al.*, *Proc. Natl. Acad. Sci. USA* 78:3629-3633 (1981). Dishes were then flooded with phosphate buffered saline (PBS) and cells recovered by centrifugation for subsequent RNA isolation and analysis.

Gene transfer efficiencies were estimated at about 60% for the Zen vector and approximately 40% for [ß-globin/LCR]^{mut} vectors. Genomic DNA from whole spleens of reconstituted animals was prepared at day 13 post-engraftment. Southern blot analysis performed subsequently showed that gene transfer into hematopoietic stem cells was obtained in all transplanted mice, as shown in Figure 2D. Correct proviral transmission with no detectable rearrangement was observed in the three mice

receiving the [HS2/ß-globin/PGK]^{mut} vector. The two mic receiving [ß-globin/(HS2 + HS3 + [4 x CP2 of HS4])/PGK]^{mut} showed different ratios of the two forms observed upon infection of cell-lines with this virus. This difference in ratio is likely to be a consequence of the oligo-clonality of bone-marrow reconstitution following engraftment, based on results obtained by Lemischka *et al.*, *Cell* 45:917-927 (1986) and Fraser *et al.*, *Proc. Natl. Acad. Sci. USA* 89:1968-1972 (1992). No additional rearranged form was detected.

Mutations are neutral for expression of the transduced ß-globin gene.

It was next determined whether extended mutagenesis was deleterious for ß-globin gene expression. RNA protection assays using appropriate probes demonstrated that mutated human ß-globin mRNA was properly initiated and spliced in infected MEL cells, as shown in Figure 6A. Mutated human ß-globin mRNA also appeared correctly initiated and spliced in cells obtained from *in vitro* clonogenic assays following infection of murine bone marrow stem cells, as shown in Figure 6B. Furthermore, the level of expression of the human ß-globin transgene in DMSO-induced MEL cells infected with [ß-globin/LCR]^{mut} viruses was similar to the gene expression level obtained with non-mutated [ß-globin/LCR] constructs electroporated into MEL cells, as shown in Figure 6A.

High and erythroid expression of the transduced human ß-globin gene in infected and DMSO-induced MEL cells.

To obtain preliminary indications on gene expression, linearized plasmids containing various [ß-globin/LCR] inserts in the context of a proviral structure were electroporated into MEL cells. Semi-adherent (APRT-) MEL cells, provided by Paul-Henri Romeo (INSERM U91, Paris, France), were grown

at 37°C with 5% CO_2/95% air in DMEM supplemented with 12% horse serum, 4.5 μg/ml glucose, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Electroporations were performed with approximately 10^7 MEL cells/ml in DMEM and 20 μg of plasmid DNA linearized with Nde1, using the Cell-porator (BRL) with the following set-up: low resistance, capacitance 1180 μF, and in the range of 250-350 V. Infections of MEL cells were performed with 3 ml of filtered supernatant of viral producers in the presence of 8 μg/ml Polybrene™ (Sigma, St. Louis, MO) as described above. Electroporated or infected MEL cells were subsequently split in medium containing 500 μg/ml (active) G418. Single or pool of resistant colonies were isolated and expended. MEL cells were induced at 37°C with 5% CO_2/95% air in DMEM supplemented with 15% fetal calf serum, 4.5 mg/ml glucose, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2% dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO) for 5 days. Total RNA was extracted by the RNAzol B method according to protocol provided by the manufacturer (Biotecx Laboratories, Inc., Houston, TX). Quantitative RNA protection assays were performed with uniformly labeled RNA probes *in vitro* transcribed with SP6 polymerase (Promega, Madison, WI) in the presence of [α-^{32}P] UTP (Amersham, Arlington Heights, IL). A human specific probe was provided by Tom Maniatis (Harvard University, Cambridge, MA): the specific protected fragment is 350 bp long and corresponds to the first and second exons of the ß-globin mRNA up to the exonic BamH1 site. A murine specific probe was constructed, so that a 145 bp fragment corresponding to the first exon of $ß_{maj}$-globin mRNA is protected. The first exons of murine $ß_{maj}$- and $_{min}$-globin genes have extended homology downstream of

the "ATG", but diverge extensively in their leader. Because of this pattern of homology and the conditions of our RNA protection assays, the murine specific probe also protects a 115 bp fragment of the murine β_{min}-globin mRNA. RNA protection assay was performed as described by Sambrook *et al.*, *Molecular cloning; a laboratory manual - 2nd ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) and with the following conditions: 10 µg of total RNA, greater than 5 x 10^5 cpm of each probe in separate reactions, hybridization at 52°C for 16 hours in [40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% formamide], digestion with 20 µg/ml RNase A (Sigma, St. Louis, MO) and 2 µg/ml RNase T1 (Sigma) for 30 minutes at room temperature. Under these conditions, sparse mismatches, such as those present in the mutated human ß-globin gene or between homologous regions of murine β_{maj}- and β_{min}- globin mRNA, are not detected. Radioactive bands corresponding to the specific protected fragments were scanned using a Phosphor Imager (Molecular Dynamics, Evry Cedex, France), and/or autoradiograms were analyzed with a 2202 Ultrascan laser densitometer (LKB Instruments, Inc., Gaithersburg, MD). Human ß-globin/murine ß-globin mRNA ratios were corrected for the number of uridine residues in each probe (factor 2.5) and on a per gene basis. Although the original isolate of aneuploid semi-adherent (APRT-) MEL cells are believed to be pseudo-tetraploid (Chao *et al.*, *Cell*, 32:483-493 (1983)), Southern blot analysis suggests that the MEL cells used in this study are rather pseudo-diploid for the endogenous mouse ß-globin genes. C rrection (factor 2) was therefore applied to account for this ratio: average of two β_{maj}- and two β_{min}- globin genes and only one provirus

per cell. Global calculations were made as follows:

$$\frac{\text{Human } \beta}{\text{Murine } \beta_{maj+min}} = \frac{[\text{human } \beta\text{-globin band}] \times 2 \times 100}{[\text{murine } \beta_{maj+min}\text{-globin bands}] \times 2.5}$$

$$\frac{\text{Human } \beta}{\text{Murine } \beta_{maj}} = \frac{[\text{human } \beta\text{-globin band}] \times 2 \times 100}{[\text{murine } \beta_{maj}\text{-globin band}] \times 2.5}$$

RNA protection assays and Southern blot analysis were performed with a pool of electroporated, G418 selected, and DMSO-induced MEL cells. Results from this experiment suggested that [2 x HS2], (HS2 + HS3) and (HS2 + [2 x HS3]) derivatives do not increase ß-globin gene expression over HS2 alone; in contrast, addition of HS4 derivatives to HS2 and HS3 appeared to enhance significantly ß-globin gene expression. Accordingly, the infection study was limited to the following ß-LCR combinations: HS2, [HS2 + (4 x 23bp of HS2)], [HS2 + (4 x 23bp of HS2) + HS3], [HS2 + HS3 + HS4], and [HS2 + HS3 + (4 x CP2 of HS4)]. These various ß-LCR derivatives were inserted in the mutated vector optimized for stable proviral transmission. A control vector containing the SV40 enhancer without ß-LCR was also included. MEL cells were infected with supernatant from producers in experimental conditions providing up to one integrated provirus per cell, and were subsequently selected with G418. Pools of infected and selected MEL cells of at least 10^2 clones were DMSO-induced for five days and subsequently analyzed for human and murine ß-globin mRNA expression by RNA protection assay. Transduced human ß-globin/murine ß-globin mRNA ratios were calculated on a per gene basis, following appropriate corrections. Corrections were applied for specific activity of the probes, and on th basis that the aneuploid MEL cells appear pseudo-

diploid for the endogenous murin ß-globin genes while containing only one copy of integrated provirus per cell following infection and G418 selection. Cell-type specificity of expression was verified by infection of NIH 3T3 cells: no expression of the transduced ß-globin gene was detected. Also, expression of the transduced human ß-globin gene was low in non-induced MEL cells. Results obtained with the various constructs are presented in Figure 6A and Table I.

Another implication for gene therapy protocols of the mono-or oligo- clonality of bone marrow reconstitutions is the necessity to engraft recipients with only transduced THSC, so that complete and sustained reconstitution with infected cells in 100% of transplanted individuals is achieved. Because a significant proportion of THSC are not infected even by high titer retroviral vectors, as reported by Lemischka *et al.*, *Cell* 45:917-927 (1986); Dzierzak *et al.*, *Nature* 331:35-41 (1988); Karlsson *et al.*, *Proc. Natl. Acad. Sci. USA* 85:6062-6066 (1988); Bender *et al.*, *Mol. Cell. Biol.* 9:1426-1434 (1989); and Fraser *et al.*, *Proc. Natl. Acad. Sci. USA* 89:1968-1972 (1991), it may be therefore desirable to add a step of selection of infected bone marrow cells prior to transplantation. Unfortunately, enhancers/promoters driving NeoR (e.g. LTR, SV40) are believed to be repressed in THSC, as is observed with other stem cells such as embryonic stem (ES) cells, as reported by Hawley *et al.*, *Plasmid*, 22:120-131 (1989). Accordingly, internal enhancers/promoters which are not repressed in ES cells were inserted in the [ß-globin/LCR]^{mut} vectors to drive NeoR. These enhancers/promoters include F441 polyoma (F441 Py) enhancer/thymidine kinase (TK) promoter and murine phosphoglycerate kinase-1

(PGK) promoter. In addition, the Tobacco Mosaic Virus (TMV) 1 ader, which is a powerful enhancer of translation (Gallie *et al.*, *Nucleic Acids Res.*, 15:3257-3273 (1987)), was added in some constructs.

Titers of these different vectors upon G418 selection of infected 3T3 cells were compared. NIH 3T3 cells, provided by Jane-Jane Chen (Harvard-MIT HST, MIT, Cambridge, MA), were grown at 37°C with 5% CO_2/95% air in DMEM supplemented with 10% calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. NIH 3T3 cells were infected with various dilutions of filtrated viral supernatant in the presence of 8 µg/ml Polybrene™ (Sigma, St. Louis, MO) as described by Danos and Mulligan, *Proc. Natl. Acad. Sci. USA* 85:6460-6464 (1988). Cells were subsequently split in medium containing 500 µg/ml (active) G418. Resistant colonies were counted, and titers estimated by standard calculations previously described (Danos and Mulligan, *supra* 1988). Proviral transmission was tested by Southern analysis (Sambrook *et al.*, *Molecular cloning: a laboratory manual - 2nd ed.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), with neoR and ß-globin specific probes and appropriate controls.

Titers were found similar for SV40, F441 Py/TK, and F441 Py/TK/TMV. In contrast, PGK increased viral titer by over five times as compared to the first group (Table I). Because these heterologous enhancers are positioned in [ß-globin/LCR]^{mut} vectors next to LCR derivatives, possible influence exerted by these heterologous enhancers on ß-LCR derivatives for transcription of the transduced ß-globin gene was also investigated. Results of this experiment indicate that heterologous enhancers are not neutral for ß-globin expression when linked to HS2. The global level of

enhancement provided by the combination [HS2 + heterologous nhancer] incr ases in the following order: SV40, PGK, and F441 Py/TK (Figure 6C and Table I).

ß-globin expression is partially independent of the sites of chromosomal integration.

Since mono- or oligo-clonality is frequently observed in long-term reconstituted hematopoietic systems (Lemischka *et al.*, *Cell* 45:917-927 (1986); Fraser *et al.*, *Proc. Natl. Acad. Sci. USA* 89:1968-1972 (1991)), it is essential to obtain expression of the transduced ß-globin gene relatively independently of the site of chromosomal integration, so that consistent and sustained ß-globin gene expression is achieved. Grosveld *et al.*, *Cell*, 51:975-985 (1987); Collis *et al.*, *EMBO J.* 9:233-240 (1990); Philipsen *et al.*, *EMBO J.* 9:2159-2167 (1990); Talbot *et al.*, *EMBO J.* 9:2169-2178 (1990); and Pruzina *et al.*, *Nucleic Acids Res.* 19:1413-1419 (1991) have reported that each of the HS sites, individually or in association, are able to confer position-independence in MEL cells and transgenic mice, although incomplete position-independence has been also reported by Curtin *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:7082-7086 (1989); Forrester *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:5439-5443 (1989); Ryan *et al.*, *Genes Dev.*, 3:314-323 (1989); and Novak *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:3386-3390 (1990). Accordingly, the variability of ß-globin mRNA expression in six independent MEL cell clones, following infection and G418 selection, was tested. [ß-globin/HS2/PGK]^{mut} was the focus of the study, based on the assumption that position independence would be reinforced by additional HS fragments if it was observed with one isolated HS site.

Complete position-independence was not observed, but the variation appears relatively moderate, as shown by Figure 6D and Table II.

Table II: Variability of expression of the transduced human ß-globin gene in independent MEL cell clones infected with [ß-globin/HS2/PGK]$_{mut}$.

Clone numbers	Human ß / Murine $ß_{maj+min}$	Human ß / Murine $ß_{maj}$	Murine $ß_{maj}$ / Murine $ß_{min}$
1	32%	70%	0.83
2	48%	108%	0.81
3	34%	89%	0.60
4	40%	89%	0.82
5	26%	59%	0.82
6	21%	54%	0.65

Independent clones following infection and G418 selection. Corrected values for specific activities of the probes and on a per gene basis (one proviral copy per cell in pseudo-diploid MEL cells).

Treatment of ß-globin disorders by gene transfer

The retroviral vector described herein is useful for the treatment of ß-globin disorders such as ß-thalassemias and sickle cell anemia by gene transfer.

Gene transfer is achieved by infecting autologous totipotent hematopoietic stem cells (THSC) with the retroviral vector in accordance with methods known to those skilled in the art.

Modifications and variations of the present invention, retroviral vectors for transducing human ß-globin gene and ß-locus control region derivatives, will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Massachusetts Institute Of Technology
(B) STREET: 77 Massachusetts Avenue
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 02139
(G) TELEPHONE: (617) 253-6966
(H) TELEFAX: (617) 258-6790

(ii) TITLE OF INVENTION: Retroviral Vectors for Transducing Beta-Globulin Gene and Beta-Locus Control Region Derivatives

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1666 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo Sapiens
(G) CELL TYPE: Beta-globin gene

(ix) FEATURE:
(A) NAME/KEY: misc_signal
(B) LOCATION: 37..298
(D) OTHER INFORMATION: /note= "Exon III"

(ix) FEATURE:
(A) NAME/KEY: misc_signal
(B) LOCATION: 299..1148
(D) OTHER INFORMATION: /note= "Intron 2"

(ix) FEATURE:
(A) NAME/KEY: misc_signal
(B) LOCATION: 1149..1370
(D) OTHER INFORMATION: /note= "Exon II"

(ix) FEATURE:
(A) NAME/KEY: misc_signal
(B) LOCATION: 1371..1501
(D) OTHER INFORMATION: /note= "Intron 1"

(ix) FEATURE:
(A) NAME/KEY: misc_signal
(B) LOCATION: 1502..1643
(D) OTHER INFORMATION: /note= "Exon I"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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TAGGGAACAA AGGAACCTTT AATAGAAATT GGACAGCAAG AAAGCGAGCT TAGTGATACT 180

TGTGGGCCAG GGCATTAGCC ACACCAGCCA CCACTTTCTG ATAGGCAGCC TGCACTGGTG 240

GGGTGAATTC TTTGCCAAAG TGATGGGCCA GCACACAGAC CAGCACGTTG CCCAGGAGCT 300

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GAAATATTGC TATTGCCTTA ACCCAGAAAT TATCACTGTT ATTCTTTAGA ATGGTGCAAA 540

GAGGCATGAT ACATTGTATC ATTATTGCCC TGAAAGAAAG AGATTAGGGA AAGTATTAGA 600

AATAAGATAA ACAAAAAAGT ATATTAAAAG AAGAAAGCAT TTTTTAAAAT TACAAATGCA 660

AAATTACCCT GATTTGGTCA ATATGTGTAC ACATATTAAA ACATTACACT TTAACCCATA 720

AATATGTATA ATGATTATGT ATCAATTGAA AATAAAAGAA AATAAAGTAG GGAGATTATG 780

AATATGCAAA TAAGCACACA TATATTCCAA ATAGTAATGT ACTAGGCAGA CTGTGTAAAG 840

TTTTTTTTTA AGTTACTTAA TGTATCTCAG AGATATTTCC TTTTGTTATA CACAATGTTA 900

AGGCATTAAG TATAATAGTA AAAATTGCGG AGAAGAAAAA AAAAGAAAGC AAGAATTAAA 960

CAAAAGAAAA CAATTGTTAT GAACAGCAAA TAAAAGAAAC TAAAACGATC CTGAGACTTC 1020

CACACTGATG CAATCATTCG TCTGTTTCCC ATTCTAAACT GTACCCTGTT ACTTCTCCCC 1080

TTCCTATGAC ATGAACTTAA CCATAGAAAA GAAGGGGAAA GAAAACATCA AGGGTCCCAT 1140

AGACTCACCC TGAAGTTCTC AGGATCCACG TGCAGCTTGT CACAGTGCAG CTCACTCAGT 1200

GTGGCAAAGG TGCCCTTGAG GTTGTCCAGG TGAGCCAGGC CATCACTAAA GGCACCGAGC 1260

ACTTTCTTGC CATGAGCCTT CACCTTAGGG TTGCCCATAA CAGCATCAGG AGTGGACAGA 1320

TCCCCAAAGG ACTCAAAGAA CCTCTGGGTC CAAGGGTAGA CCACCAGCAG CCTAAGGGTG 1380

GGAAAATAGA CCAATAGGCA GAGAGAGTCA GTGCCTATCA GAAACCCAAG AGTCTTCTCT 1440

GTCTCCACAT GCCCAGTTTC TATTGGTCTC CTTAAACCTG TCTTGTAACC TTGATACCAA 1500

CCTGCCCAGG GCCTCACCAC CAACTTCATC CACGTTCACC TTGCCCCACA GGGCAGTAAC 1560

GGCAGACTTC TCCTCAGGAG TCAGGTGCAC CATGGTGTCT GTTTGAGGTT GCTAGTGAAC 1620

ACAGTTGTGT CAGAAGCAAA TGTAAGCAAT AGATGGCTCT GCCCTG 1666

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(G) CELL TYPE: Beta-globin gene

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATTTATATGC AGAAATATT 19

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(G) CELL TYPE: Beta-globin gene

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGAGAAGAAA AAAAAAGAAA G 21

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(G) CELL TYPE: Beta-globin gene

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGAAAAGAAG GGGAAAGAAA A

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 83 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(G) CELL TYPE: Beta-globin gene

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 22..23
(D) OTHER INFORMATION: /note= "Substituted base or introduced base"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 56..60

(D) OTHER INFORMATION: /note= "Substituted bases or introduced bases"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTGTGGGAGG AAGATAAGAG GGATGAACAT GATTAGCAAA AGGGCCTAGC TTGGACGCGT 60

CATCAAGGGT CCCATAGACT CAC 83

We claim:

1. A method of making a retroviral vector for transducing ß-like globin gene and ß-LCR derivatives comprising the steps of:

(a) providing a retroviral vector in combination with a ß-globin gene derivative and an effective portion of the HS2 enhancer of the ß-LCR, with or without additional ß-LCR HS2 sites, to achieve transduction and expression of the ß-globin gene derivative, and

(b) modifying a DNA sequence of the second intron of the ß-globin gene, any complementary/reverse splice-signals, any polyadenylation signals, or combinations thereof, of the transduced ß-globin gene derivative or ß-LCR, to form a retroviral vector characterized by:

i) stability of proviral transmission upon infection of cell-lines and murine bone marrow cells,

ii) viral titer effective to achieve infection of bone marrow cells, and

iii) high erythroid expression of the transduced human β-globin gene.

2. The method of claim 1 wherein the effective viral titer is greater than 10^5 resistant colonies per ml of viral supernatant under standard conditions.

3. The method of claim 1 wherein the high erythroid expression is greater than 50% of a human ß-globin to murine $ß_{maj}$-globin mRNA ratio.

4. The method of claim 1 wherein said retroviral vector comprises:

(a) a left and right long terminal repeat (LTR),

(b) a tRNA primer binding site for initiation of synthesis of viral minus strand strong-stop;

(c) a polypurine track primer binding site for initiation of synthesis of viral plus strand strong-stop, and

(d) a packaging signal.

5. The method of claim 4 wherein said packaging signal extends into the gag region.

6. The method of claim 1 wherein said retroviral vector is a splicing vector, comprising functional splicing signals leading to genomic and sub-genomic transcripts.

7. The method of claim 1 wherein said retroviral vector is not a splicing vector.

8. The method of claim 1 wherein said retroviral vector has a selectable marker.

9. The method of claim 1 wherein said modifications are selected from the group consisting of deletions, additions, and substitutions of nucleotides in the DNA sequence of the second intron of the ß-globin gene, complementary splice-signals or polyadenylation signals of the transduced ß-globin gene or LCR.

10. The method of claim 8 wherein said selectable marker is driven by an internal enhancer/promoter.

11. The method of claim 10 wherein said selectable marker is driven by the left LTR.

12. The method of claim 8 wherein said selectable marker is placed in a splicing retroviral vector.

13. The method of claim 8 wherein said selectable marker is selected from the group consisting of a neomycin/G418 resistance gene, a hygromycin resistance gene, a puromycin resistance gene, a phleomycin resistance gene, a dihydrofolate reductase gene, a multidrug-resistance gene, and a gene for an enzyme.

14. The method of claim 8 wherein said selectable marker is a molecule that interacts with a substrate to produce a colored cell.

15. The method of claim 8 wherein said selectable marker is a molecule expressed at the cell membrane.

16. The method of claim 14 wherein said selectable marker is the gene encoding β-galactosidase.

17. The method of claim 4 wherein said right LTR comprises a deletion in the U3 region yielding a "self-inactivating" vector upon reverse transcription.

18. The method of claim 1 wherein said ß-globin gene is modified within the second intron of the ß-globin gene, while maintaining correct splicing of this intron as well as normal expression of the ß-globin transgene as compared to the non-deleted Intron 2 containing ß-globin gene.

19. The method of claim 1 wherein said transduced ß-globin gene or ß-LCR derivatives contain partial deletions, substitutions, mutations or modifications of at least one of the complementary/reverse 5′ splice-sites or 3′ splice-sites or branchpoint signals or polyadenylation signals.

20. The method of claim 1 wherein stability of proviral transmission is obtained by modifying the location, position, orientation of any of the ß-LCR segments.

21. The method of claim 1 wherein an HS2 derivative is incorporated, in a single or duplicated form or in association with other ß-LCR derivatives or heterologous enhancer sequences, in any position or orientation.

22. The method of claim 1 further comprising incorporation of an HS1 derivative in a single or duplicated form or in association with other ß-LCR derivatives or heterologous enhancer sequences, in any position or orientation.

23. The method of claim 1 wherein an HS3 derivative is incorporated, in a single or duplicated form or in association with other ß-LCR derivatives or heterologous enhancer sequences, in any position or orientation.

24. The method of claim 1 wherein an HS4 derivative is incorporated, in a single or duplicated form or in association with other ß-LCR derivatives or heterologous enhancer sequences, in any position or orientation.

25. A retroviral vector for transducing ß-like globin gene and ß-LCR derivatives comprising:

(a) a left and a right long terminal repeat (LTR),

(b) a tRNA primer binding site for initiation of synthesis of viral minus strand strong-stop,

(c) a polypurine track primer binding site for initiation of synthesis of viral plus strand strong-stop,

(d) a packaging signal,

(e) a ß-globin gene derivative, and

(f) an effective portion of the HS2 enhancer of the ß-LCR to achieve transduction and expression of the ß-globin gene derivative,

wherein the DNA sequence of the second intron of the ß-globin gene is modified, any complementary/reverse splice-signals or

polyadenylation signals of the transduced ß-globin gene derivative or ß-LCR are modified, or both are modified, in such a way that the retroviral vector exhibits stability of proviral transmission upon infection of cell-lines and murine bone marrow cells, viral titer effective to achieve infection of bone marrow cells, and high erythroid expression of the transduced human ß-globin gene.

26. The retroviral vector of claim 25 wherein the effective viral titer is greater than 10^5 resistant colonies per ml of viral supernatant under standard conditions and the high erythroid expression is greater than 50% of a human ß-globin to murine $ß_{maj}$-globin mRNA ratio.

27. The retroviral vector of claim 25 wherein said packaging signal extends into the gag region.

28. The retroviral vector of claim 25 wherein said retroviral vector is a splicing vector comprising functional splicing signals leading to genomic and sub-genomic transcripts.

29. The retroviral vector of claim 25 wherein said retroviral vector further comprises a selectable marker.

30. The retroviral vector of claim 25 wherein said modifications are selected from the group consisting of deletions, additions, and substitutions of nucleotides in the DNA sequence of the second intron of the ß-globin gene, complementary splice-signals or polyadenylation signals of the transduced ß-globin gene or LCR.

31. The retroviral vector of claim 25 wherein said selectable marker is driven by an internal enhancer/promoter.

32. The retroviral vector of claim 25 wherein said selectable marker is driven by the left LTR.

33. The retroviral vector of claim 25 wherein said selectable marker is placed in a splicing retroviral vector.

34. The retroviral vector of claim 25 wherein said selectable marker is selected from the group consisting of a neomycin/G418 resistance gene, a hygromycin resistance gene, a puromycin resistance gene, a phleomycin resistance gene, a dihydrofolate reductase gene, a multidrug-resistance gene, and a gene for an enzyme.

35. The retroviral vector of claim 25 wherein said selectable marker is a molecule that interacts with a substrate to produce a colored cell.

36. The retroviral vector of claim 23 wherein said selectable marker is a molecule expressed at the cell membrane.

37. The retroviral vector of claim 35 wherein said selectable marker is the gene encoding β-galactosidase.

38. The retroviral vector of claim 25 wherein said right LTR comprises a deletion in the U3 region yielding a "self-inactivating" vector upon reverse transcription.

39. The retroviral vector of claim 25 wherein said ß-globin gene is modified within the second intron of the ß-globin gene, while monitoring correct splicing of this intron as well as normal expression of the ß-globin transgene as compared to the non-deleted Intron 2 containing ß-globin gene.

40. The retroviral vector of claim 25 wherein said transduced ß-globin gene or ß-LCR derivatives contain partial deletions, substitutions, mutations or modifications of at least one of the complementary/reverse 5′ splice-sites or 3′ splice-sites or branchpoint signals or polyadenylation signals.

41. The retroviral vector of claim 25 wherein stability of proviral transmission is obtained by modifying the location, position, orientation of any of the ß-LCR segments.

42. The retroviral vector of claim 25 further comprising a derivative selected from the group consisting of an HS1 derivative, an HS2 derivative, an HS3 derivative, an HS4 derivative, or combinations thereof, in a single or duplicated form or in association with other ß-LCR derivatives or heterologous enhancer sequences, in any position or orientation.

43. A method of treating a human genetic disorder of the ß-globin gene by gene therapy comprising

incubating cells from a patient suffering from the disorder with a retroviral vector capable of stable transduction of ß-like globin gene and ß-Locus control Region derivatives,

wherein the retroviral vector infects bone marrow cells, transduces the human ß-globin gene and ß-Locus control Region derivatives, and causes high erythroid expression of the transduced human ß-globin gene.

44. The method of claim 43 wherein the retroviral vector comprises:

(a) a left and a right long terminal repeat (LTR),

(b) a tRNA primer binding site for initiation of synthesis of viral minus strand strong-stop,

(c) a polypurine track primer binding site for initiation of synthesis of viral plus strand strong-stop,

(d) a packaging signal,

(e) a ß-globin gene derivative, and

(f) an effective portion of the HS2 enhancer of the ß-LCR to achieve transduction and expression of the ß-globin gene derivative,

wherein the DNA sequence of the second intron of the ß-globin gene is modified, any complementary/reverse splice-signals or polyadenylation signals of the transduced ß-globin gene derivative or ß-LCR are modified, or both are modified, in such a way that the retroviral vector exhibits stability of proviral transmission upon infection of cell-lines and murine bone marrow cells, viral titer effective to achieve infection of bone marrow cells, and high erythroid expression of the transduced human ß-globin gene.

45. The method of claim 43 wherein the genetic disorder is selected from the group consisting of ß-thalassemias and sickle cell anemia.

46. A method of transducing ß-like globin gene and ß-LCR derivatives comprising infecting a cell with a retroviral vector comprising:

(a) a left and a right long terminal repeat (LTR),

(b) a tRNA primer binding site for initiation of synthesis of viral minus strand strong-stop,

(c) a polypurine track primer binding site for initiation of synthesis of viral plus strand strong-stop,

(d) a packaging signal,

(e) a ß-globin gene derivative, and

(f) an effective portion of the HS2 enhancer of the ß-LCR to achieve transduction and expression of the ß-globin gene derivative,

wherein the DNA sequence of the second intron of the ß-globin gene is modified, any complementary/reverse splice-signals or polyadenylation signals of the transduced ß-globin gene derivative or ß-LCR are modified, or both are modified, in such a way that the retroviral vector exhibits stability of proviral transmission upon infection of cell-lines and murine bone marrow

cells, viral titer effective to achieve infection of bone marrow cells, and high erythroid expression of the transduced human ß-globin gene.

47. The method of claim 46 wherein the infected cell is a bone marrow cell.

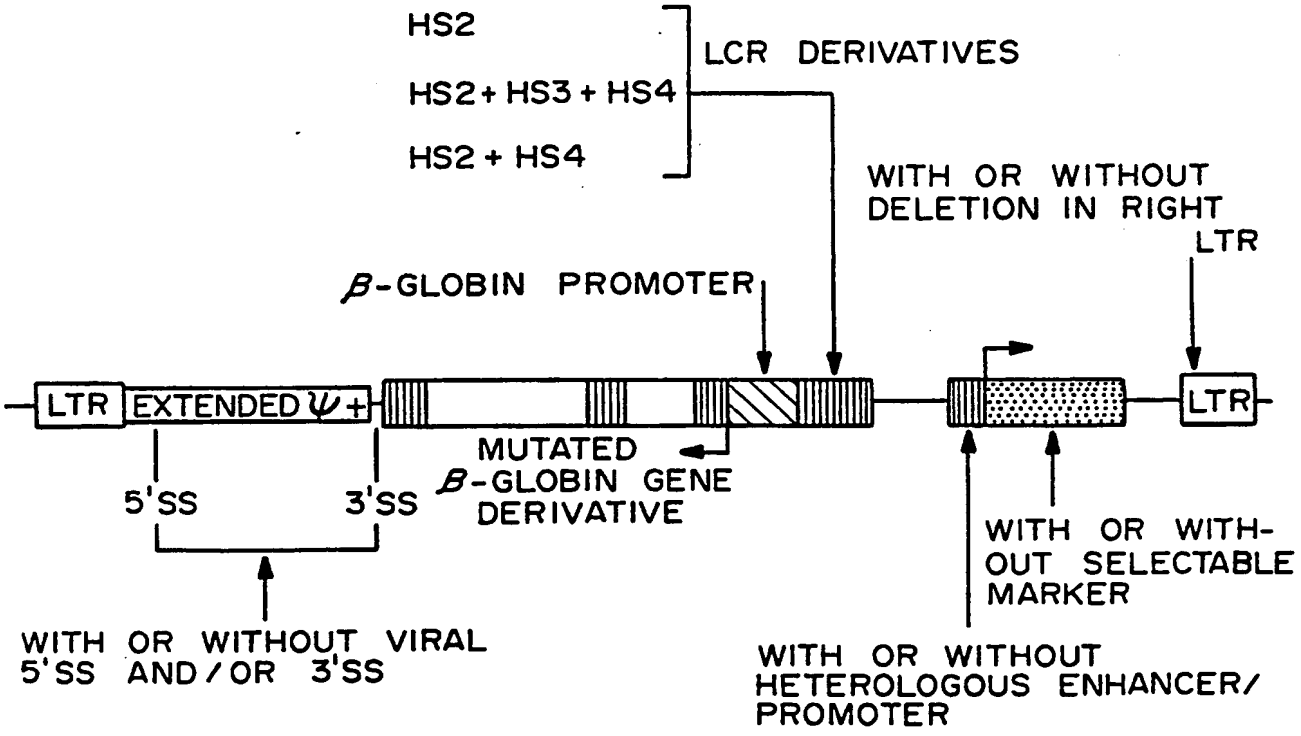

HS2
HS2+HS3+HS4
HS2+HS4
LCR DERIVATIVES
WITH OR WITHOUT DELETION IN RIGHT LTR
β-GLOBIN PROMOTER
LTR
EXTENDED Ψ+
LTR
MUTATED β-GLOBIN GENE DERIVATIVE
5'SS
3'SS
WITH OR WITHOUT VIRAL 5'SS AND/OR 3'SS
WITH OR WITH-OUT SELECTABLE MARKER
WITH OR WITHOUT HETEROLOGOUS ENHANCER/ PROMOTER

FIG. 1

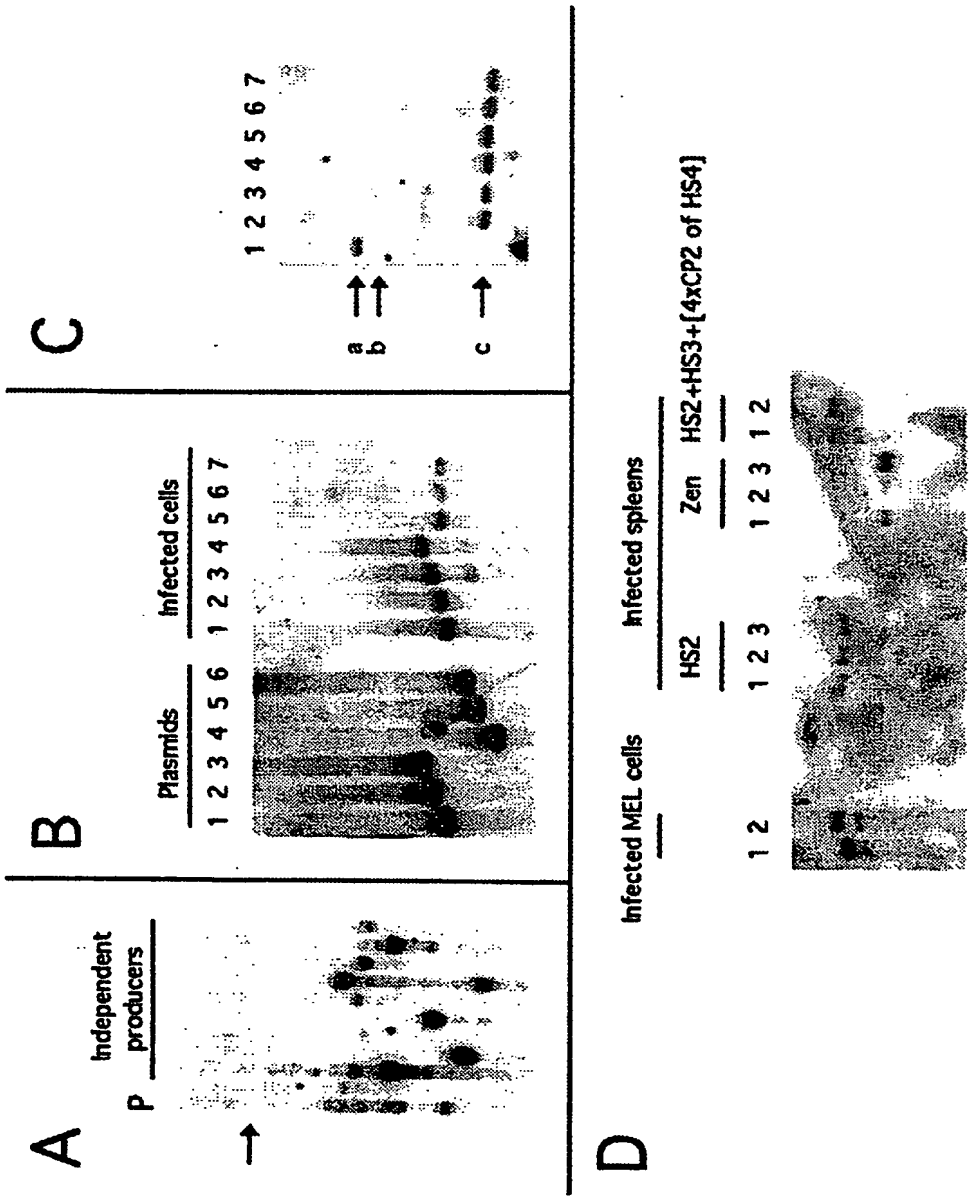

A
Independent producers
P
B
Plasmids
1 2 3 4 5 6
Infected cells
1 2 3 4 5 6 7
C
1 2 3 4 5 6 7
a
b
c
D
Infected MEL cells
1 2
Infected spleens
HS2
1 2 3
Zen
1 2 3
HS2+HS3+(4xCP2 of HS4)
1 2

FIGURE 2

	5' SS	BPS + 3'SS		Poly A
1	AGGTGAGc (III)			
325		TTgGAa	TCTTgTCTgCTGCAG	
406		gCCaGAC	TaCCaCTCCCTTaAG	
430	AGGTcAcT			
501		TGTcAAg	CCTTCTgCTCTGCAG	
774		CCTcGAT	CCTCCCTTTaTCCAG	
792		CCTcGAT	TCaCTCCTTCTCTAG	
* 847	AGGTGgtT			
* 1048		TACaAAa	TCaaaTTCTTCTCAG	
* 1084		TACTAAg	CCagTCCTTCCAaAG	
* 1153	ctGTGAGT			
* 1212		gTCaAAC	TTTTCCTTCTTCaAG	
* 1215		gTCaAAC	TCCTTCTTCaaGTAG	
* 1558		TTTgcAg	CCTTCTTTCaTGgAG	
* 1648		CACTGAC	aCaTTCCCTTTTTAG	
* 1704				AATAAA+[T]n
* 1712		aATaAAT	TTTTTTaTTagGCAG	
* 1754		TCCaGAa	TaTCCCCCagTTTAG	
* 1756		TTCatAa	CCCCCagTTTaGTAG	
1868		gCCacAC	aCCaCTTTCTgATAG	
1899	gGGTGAaT			
1975	AGGTAtGa (I)			
2044				AATAAA
* 2173		CCTTAAC	aCTgTTaTTCTTTAG	
* 2409				AATAAA
* 2419				AATAAA
* 2647				AATAAA
2886	AGGTGAGc (III)			
2920		CACTAAa	CTTTCTTgCCaTgAG	
3100		aACccAa	TCTCCaCaTgCCCAG	
* 3195		CACcAAC	CaCCTTgCCCCACAG	
* 3221		gCCccAC	CagaCTTCTCCTCAG	
* 3224		gCCccAC	aCTTCTCCTCaGgAG	
3298	AtGTAAGc			

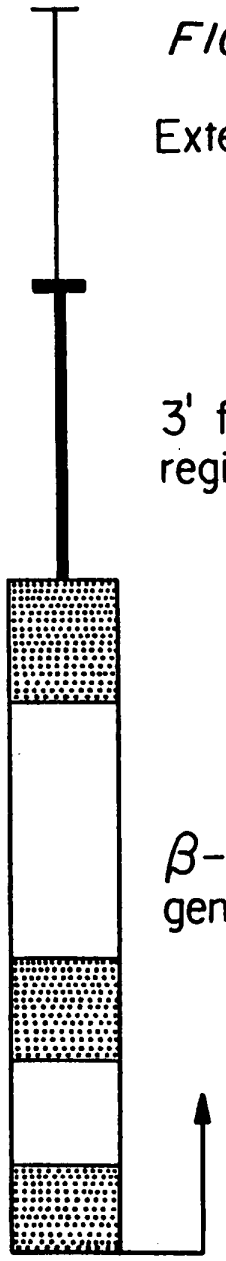
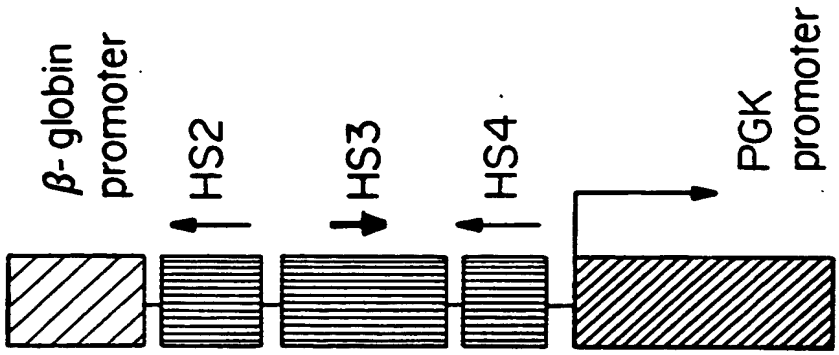


FIG. 3a

FIG. 3b



	5'SS	BPS	3'SS	Poly A
3355	gGGTGAGg (III)	TGCccAg	CCCTgCTCCTgGgAG	
3402		TTCaAAC	agCCCTCCCTCTaAG	
3481		TACaAAT	aTCCTCCTTTgCaAG	
3543				
3578	AGGTcAcT	TATTtAC	CaCaTTCTgTCTCAG	
3889				
3956	cGGTGAcT			
4079	AGGTGgtT			
4087	AGGTcAGg			
4092	AGGTtgGT (IV)			
4171	AGGTGgGg			
4286	gaGTGAGT	TCTgGAg	gCCCTggCTCTGCAG	
4349				
4529	AGGTcAaT	TCTaGAT	TCCCCCgggCTGCAG	
4554		aTTctAC	CTTTTCCCaagGCAG	
4582		no BPS	CTaCTCCTCCCCTAG	
4728		no BPS	TCCTCCCCTagTCAG	
4732		CCCctAg	CCCCCCgCCCCGCAG	
4754				
4924	gGGTGgGT	TCCTcAT	gCCTTTCggCTGCAG	
5065				

FIG. 4a

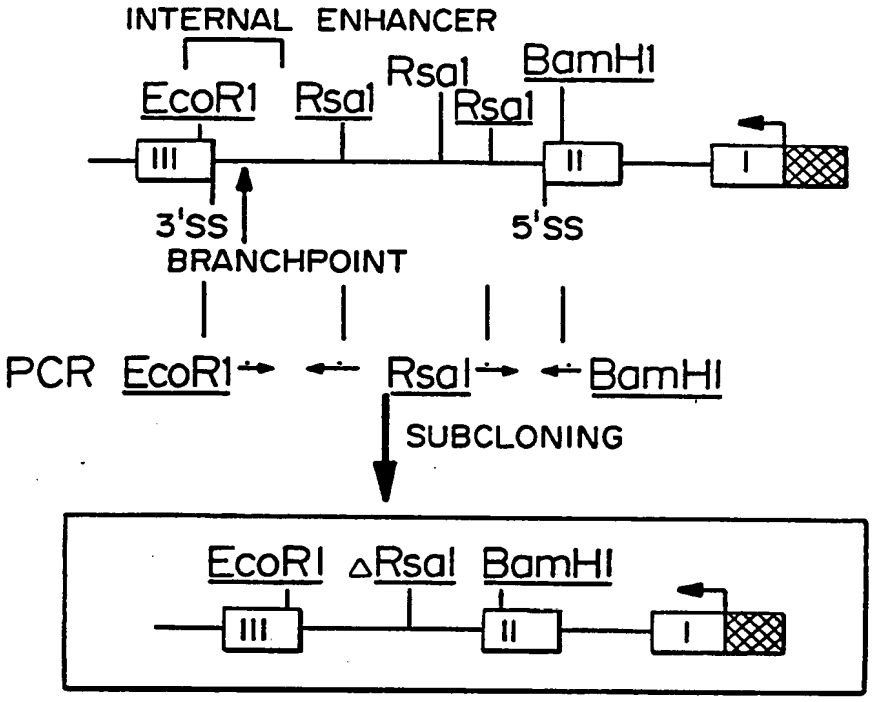

INTERNAL ENHANCER
EcoR1
RsaI
RsaI
RsaI
BamHI
III
II
I
3'SS
5'SS
BRANCHPOINT
PCR EcoR1 RsaI BamHI
SUBCLONING
EcoR1 ΔRsaI BamHI
III
II
I

FIG. 4b

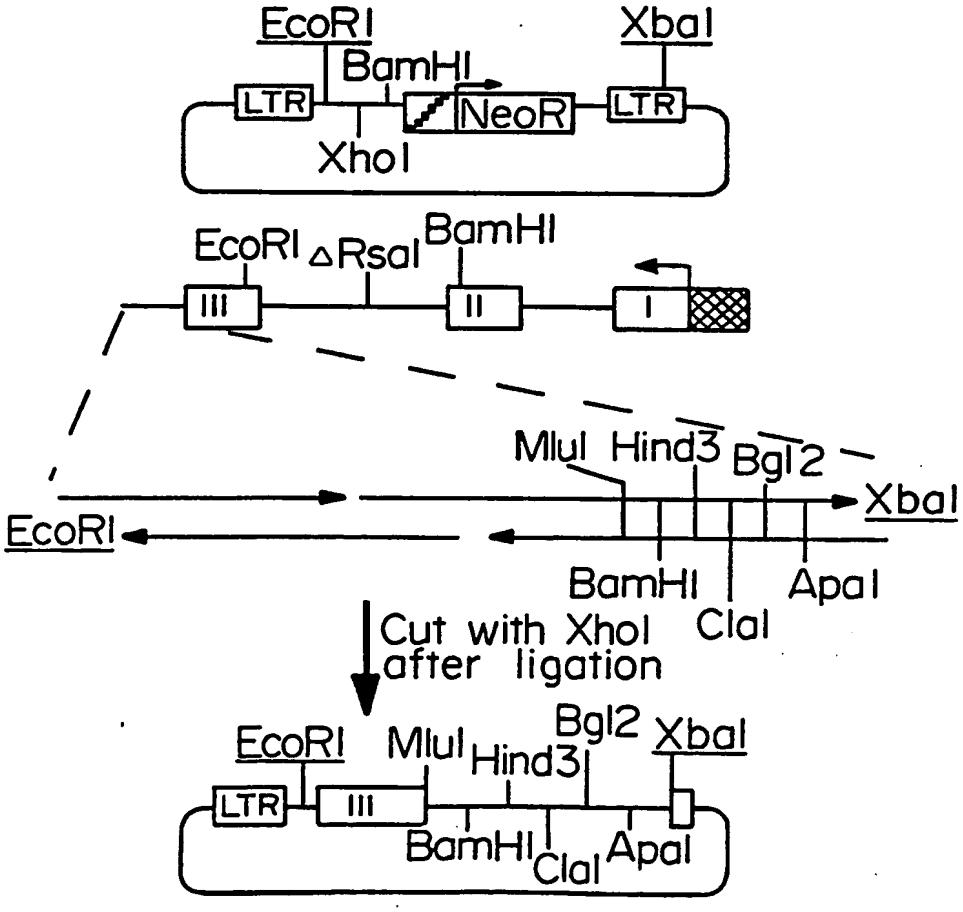

EcoR1
XbaI
BamHI
LTR
NeoR
LTR
XhoI
EcoR1 ΔRsaI BamHI
III
II
I
MluI Hind3 Bgl2
XbaI
EcoR1
BamHI
ApaI
ClaI
Cut with XhoI
after ligation
EcoR1
MluI
Hind3
Bgl2
XbaI
LTR
III
BamHI
ClaI
ApaI

FIG. 4c

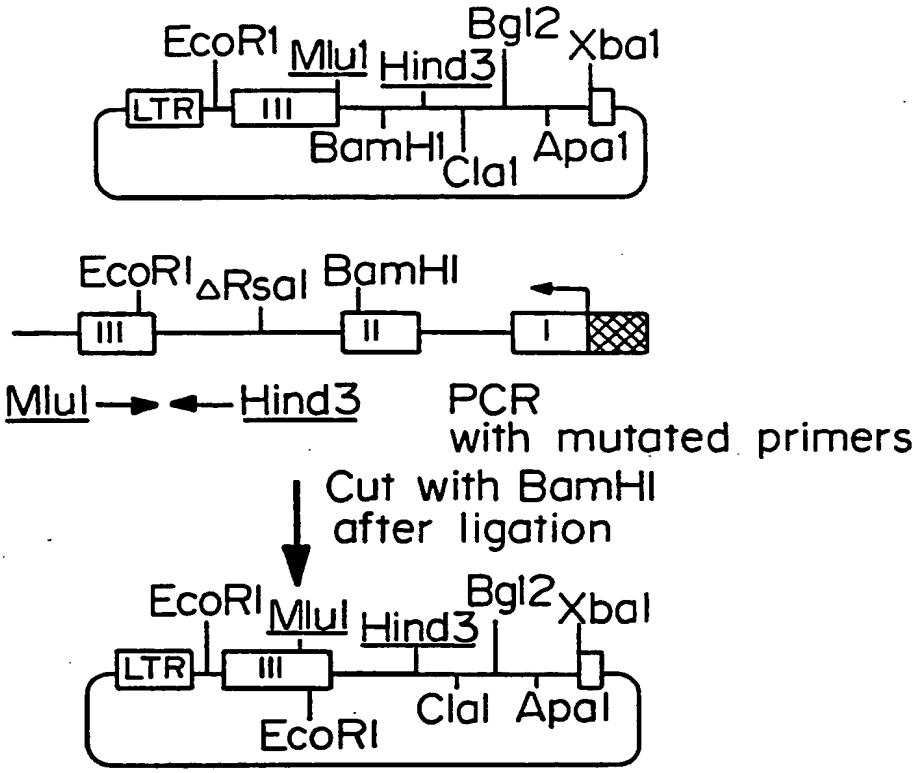

EcoR1
MluI
Hind3
Bgl2
Xba1
LTR
III
BamHI
ClaI
Apa1
EcoRI
ΔRsaI
BamHI
III
II
I
MluI
Hind3
PCR
with mutated primers
Cut with BamHI
after ligation
EcoRI
MluI
Hind3
Bgl2
Xba1
LTR
III
ClaI
Apa1
EcoRI

FIG. 4d

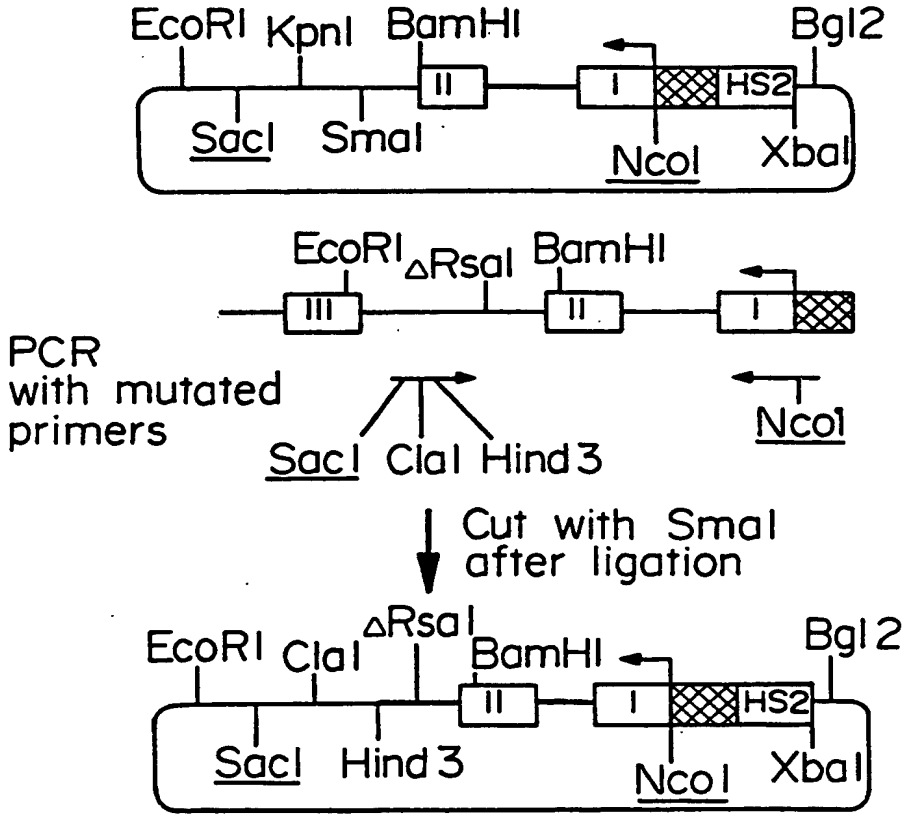

EcoRI
KpnI
BamHI
Bgl2
II
I
HS2
SacI
SmaI
NcoI
XbaI
EcoRI
ΔRsaI
BamHI
III
II
I
PCR
with mutated
primers
SacI
ClaI
Hind3
NcoI
Cut with SmaI
after ligation
EcoRI
ClaI
ΔRsaI
BamHI
Bgl2
II
I
HS2
SacI
Hind3
NcoI
XbaI

FIG. 4e

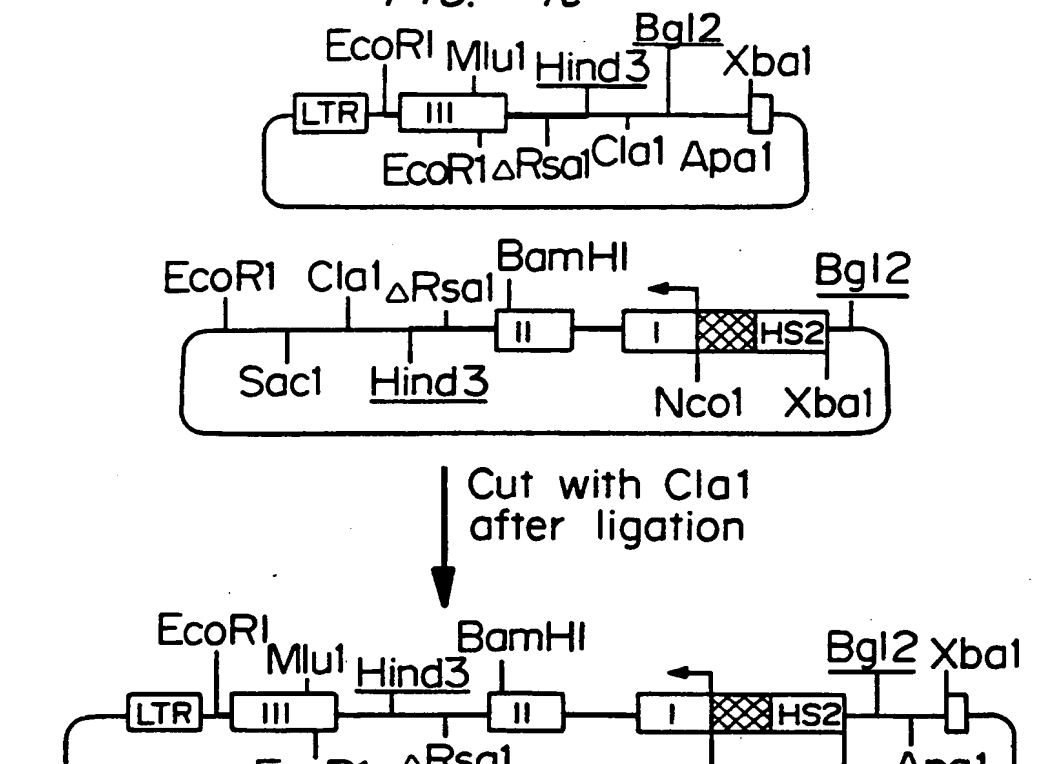

EcoRI
MluI
Hind3
Bgl2
XbaI
LTR
III
EcoR1 ΔRsaI ClaI ApaI
EcoR1 ClaI ΔRsaI
BamHI
Bgl2
II
I
HS2
SacI
Hind3
NcoI
XbaI
Cut with ClaI after ligation
EcoRI
MluI
Hind3
BamHI
Bgl2 XbaI
LTR
III
II
I
HS2
EcoR1
ΔRsaI
NcoI
XbaI
ApaI

FIG. 4f

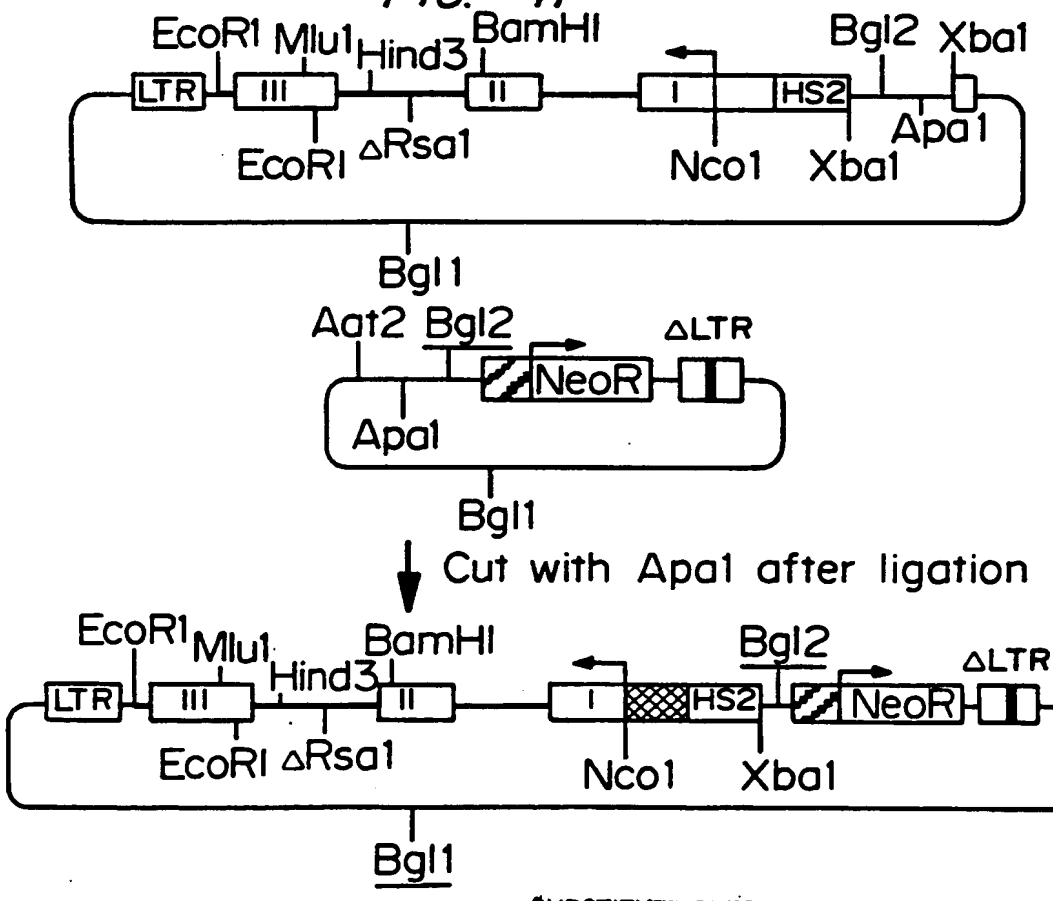

EcoR1 MluI Hind3 BamHI
Bgl2 XbaI
LTR
III
II
I
HS2
ApaI
EcoRI ΔRsaI
NcoI
XbaI
Bgl1
Aat2 Bgl2
ΔLTR
NeoR
ApaI
Bgl1
Cut with ApaI after ligation
EcoR1 MluI Hind3 BamHI
Bgl2
ΔLTR
LTR
III
II
I
HS2
NeoR
EcoRI ΔRsaI
NcoI
XbaI
Bgl1

FIG. 5a

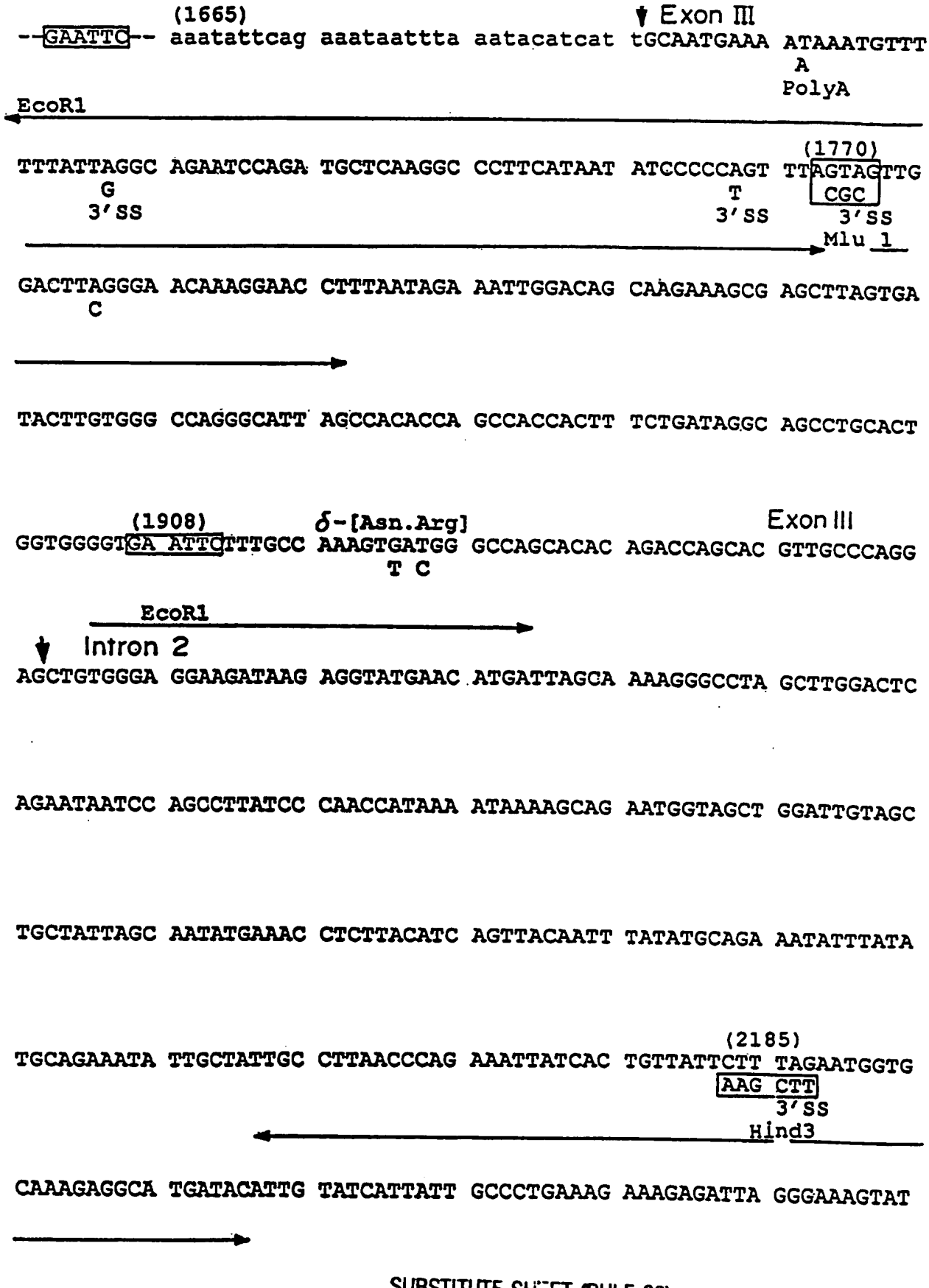

(1665)
Exon III
--GAATTC-- aaatattcag aaataattta aatacatcat tGCAATGAAA ATAAATGTTT
A
PolyA
EcoR1
(1770)
TTTATTAGGC AGAATCCAGA TGCTCAAGGC CCTTCATAAT ATCCCCCAGT TTAGTAGTTG
G T CGC
3'SS 3'SS 3'SS
Mlu 1
GACTTAGGGA ACAAAGGAAC CTTTAATAGA AATTGGACAG CAAGAAAGCG AGCTTAGTGA
C
TACTTGTGGG CCAGGGCATT AGCCACACCA GCCACCACTT TCTGATAGGC AGCCTGCACT
(1908)
δ-[Asn.Arg]
Exon III
GGTGGGGTGA ATTCTTTGCC AAAGTGATGG GCCAGCACAC AGACCAGCAC GTTGCCCAGG
T C
EcoR1
Intron 2
AGCTGTGGGA GGAAGATAAG AGGTATGAAC ATGATTAGCA AAAGGGCCTA GCTTGGACTC
AGAATAATCC AGCCTTATCC CAACCATAAA ATAAAAGCAG AATGGTAGCT GGATTGTAGC
TGCTATTAGC AATATGAAAC CTCTTACATC AGTTACAATT TATATGCAGA AATATTTATA
(2185)
TGCAGAAATA TTGCTATTGC CTTAACCCAG AAATTATCAC TGTTATTCTT TAGAATGGTG
AAG CTT
3'SS
Hind3
CAAAGAGGCA TGATACATTG TATCATTATT GCCCTGAAAG AAAGAGATTA GGGAAAGTAT

FIG. 5b

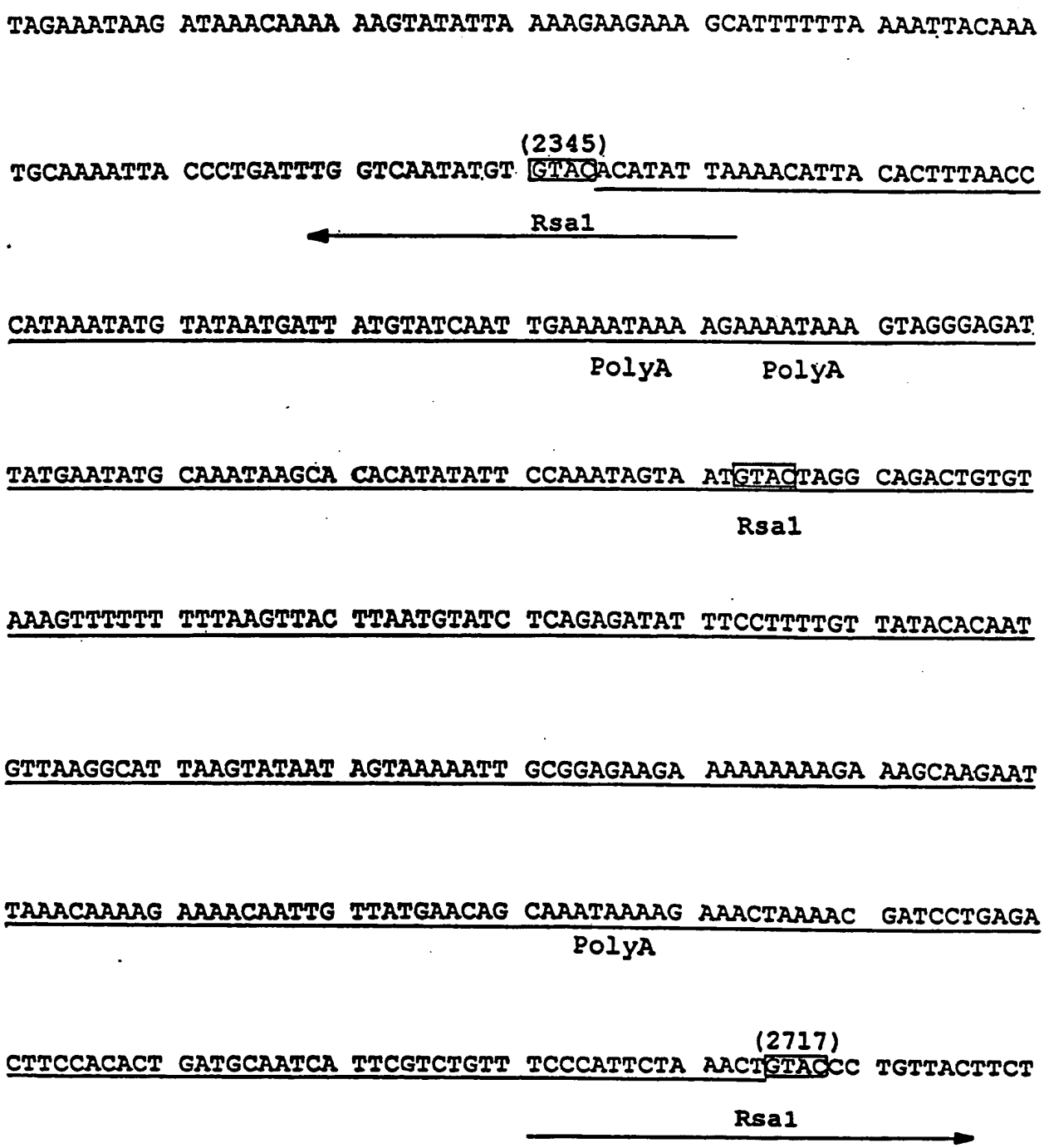
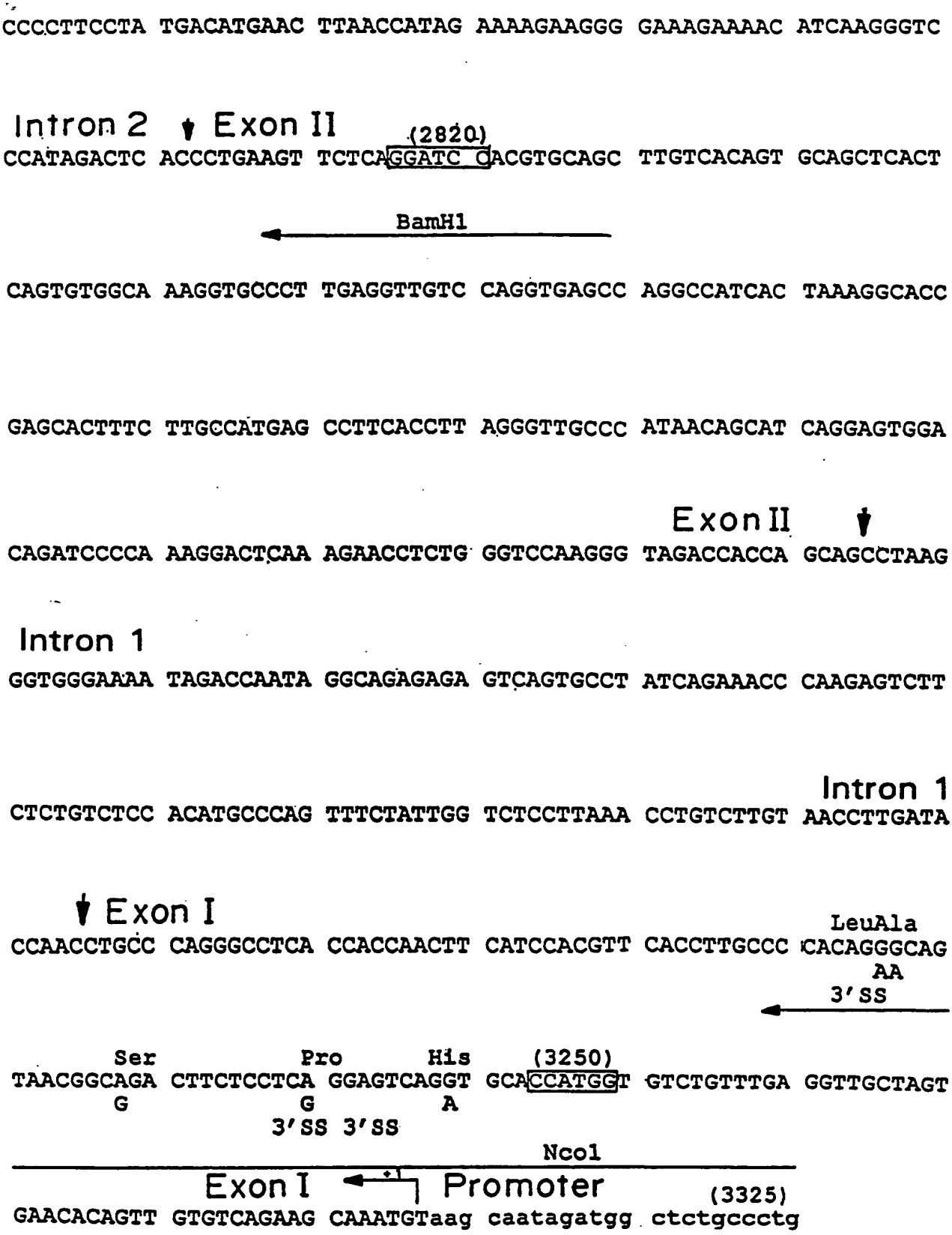

TAGAAATAAG ATAAACAAAA AAGTATATTA AAAGAAGAAA GCATTTTTTA AAATTACAAA
(2345)
TGCAAAATTA CCCTGATTTG GTCAATATGT GTACACATAT TAAAACATTA CACTTTAACC
RsaI
CATAAATATG TATAATGATT ATGTATCAAT TGAAAATAAA AGAAAATAAA GTAGGGAGAT
PolyA PolyA
TATGAATATG CAAATAAGCA CACATATATT CCAAATAGTA ATGTACTAGG CAGACTGTGT
RsaI
AAAGTTTTTT TTTAAGTTAC TTAATGTATC TCAGAGATAT TTCCTTTTGT TATACACAAT
GTTAAGGCAT TAAGTATAAT AGTAAAAATT GCGGAGAAGA AAAAAAAAGA AAGCAAGAAT
TAAACAAAAG AAAACAATTG TTATGAACAG CAAATAAAAG AAACTAAAAC GATCCTGAGA
PolyA
(2717)
CTTCCACACT GATGCAATCA TTCGTCTGTT TCCCATTCTA AACTGTACCC TGTTACTTCT
RsaI

FIG. 5c


CCCCTTCCTA TGACATGAAC TTAACCATAG AAAAGAAGGG GAAAGAAAAC ATCAAGGGTC
Intron 2
Exon II
(2820)
CCATAGACTC ACCCTGAAGT TCTCAGGATC CACGTGCAGC TTGTCACAGT GCAGCTCACT
BamH1
CAGTGTGGCA AAGGTGCCCT TGAGGTTGTC CAGGTGAGCC AGGCCATCAC TAAAGGCACC
GAGCACTTTC TTGCCATGAG CCTTCACCTT AGGGTTGCCC ATAACAGCAT CAGGAGTGGA
Exon II
CAGATCCCCA AAGGACTCAA AGAACCTCTG GGTCCAAGGG TAGACCACCA GCAGCCTAAG
Intron 1
GGTGGGAAAA TAGACCAATA GGCAGAGAGA GTCAGTGCCT ATCAGAAACC CAAGAGTCTT
Intron 1
CTCTGTCTCC ACATGCCCAG TTTCTATTGG TCTCCTTAAA CCTGTCTTGT AACCTTGATA
Exon I
LeuAla
CCAACCTGCC CAGGGCCTCA CCACCAACTT CATCCACGTT CACCTTGCCC CACAGGGCAG
AA
3'SS
Ser
Pro
His
(3250)
TAACGGCAGA CTTCTCCTCA GGAGTCAGGT GCACCATGGT GTCTGTTTGA GGTTGCTAGT
G
G
A
3'SS 3'SS
NcoI
Exon I
Promoter
(3325)
GAACACAGTT GTGTCAGAAG CAAATGTaag caatagatgg ctctgccctg

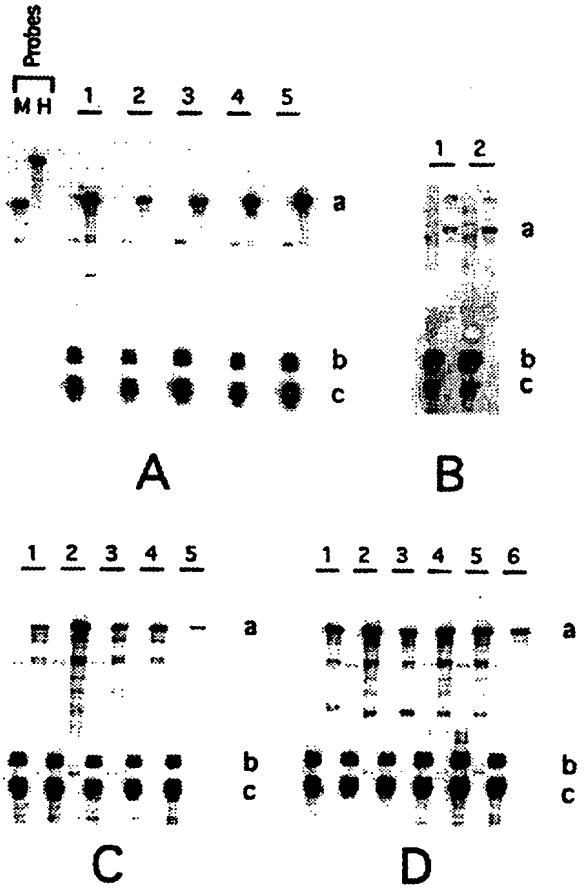


FIGURE 6

INTERNATIONAL SEARCH REPORT

Intr onal Application No
PCT/US 94/06661

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/85 C12N15/86 C07K14/805 A61K48/00 C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BONE MARROW TRANSPLANTATION, vol.9, 1992 pages 154 - 157 R. GELINAS ET AL. 'A normal level of beta globin expression in erythroid cells after retroviral cells transfer' see the whole document ---	1-6, 8-13, 18-20, 25-34, 39-47
Y		21-24
Y	BLOOD, vol.81, no.5, 1993 pages 1384 - 1392 I. PLAVEC ET AL. 'A human beta-globin gene fused to human beta-globin locus control region' see the whole document --- -/--	21-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 October 1994	- 9. 11. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Skelly, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 94/06661

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROC. NATL. ACAD. SCI. USA, vol.87, 1990 pages 3386 - 3390 U. NOVAK ET AL. 'High-level beta globin expression after retroviral transfer of locus activation region' see the whole document	21-24
A	PROC. NATL. ACAD. SCI. USA, vol.89, 1992 pages 3107 - 3110 J. CHANG ET AL. 'A 36-base pair core sequence of locus control region enhances retrovirally transferred human beta globin expression' cited in the application see the whole document	21-24
A	J. VIROL., vol.62, no.11, 1988 pages 4337 - 4345 D. MILLER ET AL. 'Design of retrovirus vectors for transfer and expression of human beta globin gene' cited in the application	
A	WO,A,89 02469 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 23 March 1989 cited in the application	1
A	WO,A,89 01517 (GROSVELD) 23 February 1989	

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/ 06661

B x I Observations where certain claims were found unsearchable (Continuation f item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 43-45 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No.
PCT/US 94/06661

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8902469	23-03-89	EP-A-	0377676	18-07-90
		JP-T-	3500245	24-01-91
		US-A-	5126260	30-06-92
WO-A-8901517	23-02-89	AU-A-	2137388	09-03-89
		EP-A-	0332667	20-09-89
		JP-T-	2500802	22-03-90